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Video-based identification of surrogate endpoints in experimental bacterial infections of rainbow trout (*Oncorhynchus mykiss*)

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Video-based identification of surrogate endpoints in experimental bacterial infections of rainbow trout (*Oncorhynchus mykiss*)

To identify visually perceptible clinical signs that would enable timely distinction between survivors and nonsurvivors in infection experiments requiring death as experimental endpoint, rainbow trout were recorded on video after being subjected to bacterial challenge with *Aeromonas salmonicida* and *Yersinia ruckeri*. Deviations from normal morphology, behaviour and movement patterns were analysed for their potential to predict death in infected animals kept in groups of 10 fish inside small volume holding tanks (15 l). It was found that clinical signs reflecting a highly debilitated physiological state, like inability to hold an upright position or being passively dragged by a current, offer high sensitivity and specificity, but are largely inefficient in reducing the overall time an animal spends inside the experiment. A change in body shape detected in fish infected with *A. salmonicida*, provided high sensitivity and earlier identification of nonsurvivors. Anorexia was identified as a promising death predictor in terms of sensitivity and timely identification, but its specificity was likely to be confounded by a high level of social aggression observed between the fish. As expression of early clinical signs in form of sickness behaviour might have been strongly influenced by experimental husbandry conditions, it is assumed that optimisation of those could present an effective approach for improving applicability of surrogate endpoints in this species.

humane endpoints - rainbow trout - death predictor - social aggression - sickness behaviour

Abbreviations and variables

Abd	Abdominal curvature
Anor	cAnorexia
AsT1	Aeromonas salmonicida Trial1
AsT2	Aeromonas salmonicida Trial2
AsT1/T2	Aeromonas salmonicida Trials
BC	Bottom contact
cAnor	Complete Anorexia
CFU	Colony forming unit
Col	Collision
DLR	Dorsal or lateral recumbency
DVax	Angle dorso-ventral axis
FD	Fin damage
Fmov	Fin movement
LiPi	Light perpendicular instability
Loc	Locomotion
MAD(·)	Median absolute deviation of (·)
MLBo	Motionless on tank bottom
MP	Marginal position
ObNa%	Percentage of non available observations
PaFlo	Passive Floating
PDP	Potential death predictor
Sens	Sensitivity
SevPi	Severe perpendicular instability
SLoc	Stiffened locomotion
Sn	Snout lesion
Spec	Specificity
StatCur	Stability in current
t_{death}	Time of death
t_{Foc}	Forecast time
t_{inf}	Time of bacterial infection
t_{Obs}	Time to first/second observation
t_{PDP}	Time of first/second observation
t_{surv}	Survival time
TAbd	Tucked-up abdomen
TSS	The stock solution
VPT	Visually perceptible trait
XQ(·)	X-quantile of (·)
50Q(·)	Median of (·)
YrT1	Yersinia ruckeri Trial1
YrT2	Yersinia ruckeri Trial2
YrT1/T2	Yersinia ruckeri Trials

Chapter 1

Introduction

In order to warrant responsible use of experimental animals in scientific research, many countries made animal welfare an integral part of their legislation [27, 19, 17]. Those legal framework conditions aim to coerce institutions into applying certain measures when conducting animal experiments. The required measures are generally being derived from the so called 3 R concept (**R**eplacement, **R**eduction and **R**efinement), introduced by Russel and Burch in 1959 [35]. This concept considers replacement of use of live animals by alternative methods as the most desirable solution. However, reduction of animal numbers to a statistically sound minimum, together with adopting measures of refinement are regarded essential requirements if replacement cannot be achieved. "Any approach which avoids or minimizes the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their wellbeing" [10] is being assigned to refinement.

One fundamental pillar of refinement is the application of so called "humane endpoints". To many animal welfare agencies, the application of humane endpoints presents an obligatory requirement for granting the permission to perform an animal experiment. Morton (1999) defines five types of humane endpoints, among them the so called "surrogate endpoint". He specifies it as pre-lethal or pre-painful endpoint that can replace a scientifically justified experimental endpoint[1] with the aim of reducing unnecessary suffering of the animals involved. In order to do so, surrogate endpoints have to occur earlier in the course of experiment, while allowing reliable prediction of those results that would have been achieved by using the original experimental endpoints. Otherwise the informative value might be diminished and realisation of the whole experiment would become ethically and economically questionable. It is this kind of endpoint that will be the subject of this text, therefore the term surrogate endpoint will be applied.

Clinical signs [2] (e.g. weight loss, certain blood parameters, body temperature, presence of paralysis or ataxia, anorexia, somnolence) are used to define surrogate endpoints. Which clinical signs emerge during the course of disease depends on the pathogenesis. Pathogenesis can vary tremendously among different experimental treatments, animal species [3] and husbandry conditions (e.g. enriched vs. non-enriched cages [24]). Surrogate endpoints should be therefore determined specifically for every scientific procedure, every species (even strain) [27] and environment. Despite of potential variation in the course of disease between individuals [44], standardised nature of modern scientific animal experimentation should offer advantagious precondition for identifying surrogate endpoints that are transferable to all animals of one species undergoing a certain procedure [28] under the same husbandry conditions.

There are a number of publications discussing theoretical and practical aspects of establishing surrogate endpoints, some examples of which should be mentioned here. In his publication from

[1] Experimental endpoint in animal experimentation generally refers to a predefined event, whose exact definition depends on the research question. In a number of vaccine potency tests, death serves as the experimental endpoint [11, 18]

[2] alternative term: biomarker [15]

[3] e.g. while dogs, rats and mice tolerate well the antiparasitic agent permethrin, the same substance is highly toxic to cats, reptiles and fish which show clinical signs in form of muscular spasms, generalised tremor and seizures

2000 Morton [27] describes how surrogate endpoints can be established and reliably applied by introduction of a score sheet system. To develop such a score sheet system, he proposes compilation of occurring clinical signs i) by very close observation of the animals undergoing the scientific procedures, and ii) by extensive exchange of information between animal care staff, veterinarians and scientists. According to Morton, this "team approach" has proven to be the best precondition for successfully identifying clinical signs that might be applicable as surrogate endpoint. The score sheet, which is developed from the list of clinical signs, should enable efficient, evidence-based evaluation of an animal's status. Rather than presenting a self-contained work, the score sheet should be continuously validated and optimised during its application. This approach is considered to be suitable for all scientific procedures and all vertebrate species (and eventually even invertebrate species [27]). One working group report by Jennings et al. (2010) gives concrete instructions on how to identify and implement surrogate endpoints in vaccine studies (including batch-potency tests, model development, proof-of-concept, challenge validation, challenge passage and efficacy studies). Instructions of how to establish surrogate endpoints, as well as propositions of clinical signs that might serve as surrogate endpoints are given by a number of guidelines (e.g OECD Guidelines [1]). Most of these working group reports and guidelines focus on terrestrial animals [17]. There exist a number of published scientific studies validating reliability and applicability of certain surrogate endpoints in specific animal experiments [12, 43, 29, 24]).

Fish, however are frequently overlooked in working group reports, for instance in the European Comission Working group report (2009) on severity classification [17]. Compared to the situation in mammals, only few guidelines and publications (e.g. [2, 26, 17]) give concrete advice on which clinical signs could constitute suitable surrogate endpoints for scientific procedures using fish. Often species specific properties are not being accounted for. Studies validating surrogate endpoints are almost non-existent in fish. The lack of specifications regarding surrogate endpoints in fish in European and North American regulatory guidelines has elicited criticism (e.g by [11, 19, 26]). Additionally, the comparably high number of fish recommended for vaccine batch potency tests in the European Pharmacopoeia has been suspected to be arbitrary and influenced rather by costs or availability than by scientific considerations [11, 26]. Compared to the situation in mammals, there seems to be little progress with respect to reduction and refinement in animal experimentation using fish. This includes vaccine potency testing [11, 26, 40]. Despite of widespread integration of the 3R in governing law, the scientific community performing infection experiments on fish is apparently facing particular difficulties, when it comes to establishing surrogate endpoints. For development and quality control of vaccines, Cooper & Jennings (2008) as well as Midtlyng et al. (2011) therefore demand more research into the identification of surrogate endpoints in fish vaccine studies.

It can be speculated, if it is indeed more difficult to establish surrogate endpoints in fish compared to other animal models. One particular feature that might complicate the identification of surrogate endpoints in fish could be the limited use of physiological indicators whose collection requires handling [26, 17]. For aquatic gill breathers, handling presents a particular strain. Exposure to air can lead to gill collapse with subsequent oxygen-deficiency and can cause stress even if no physical injury occurred [17]. Another aggravating factor when it comes to handling is the non-keratinised skin covered with a layer of mucus. The integument of fish is considerably more sensitive to mechanical injury and subsequent secondary infection than the skin of terrestrial animals. Additionally, the skins slippery nature makes fixation of a moving animal challenging. Moreover, most fish species lack any body extensions or extremities that would allow fixation and in contrast to many terrestrial mammals, no skin folds can be drawn. There are no reports about applicability and efficiency of training fish, although this has been suggested as a possible solution for fish in general [17]. Handling is therefore recommended to be kept to a minimum. The use of surrogate endpoints that require frequent handling (e.g. classical clinical examination, weighing) can be therefore considered largely infeasible for fish.

Another hampering factor might be the traditionally large group size of fish that are being

kept together in one tank [4]. Thus, keeping track of a single animal in order to check for clinical signs can be considered tedious and too time consuming for a human observer [39, 26], especially when the animals are excited and move quickly though the tank, which is usually the case during feeding. Overlooking a single animal that demonstrates clinical signs, especially if those are more subtle, can be considered likely under these circumstances. Identification of individual animals and tracking their health status over time is usually not warranted during a standard fish infection experiment with group size larger than n=1, due to high morpholigical similarity. Marking fish presents a challenge. Especially with small animal size and when tagging should be recognised from a distance without handling, choice of methods is highly limited [5]. The large numbers of animals used in a single trial might also prevent development of surrogate endpoints by the fact, that application of surrogate endpoints can be considered more costly in terms of workload and costs. To record death requires little time, is objective and easy to standardize and requires little training [18]. For example, applying surrogate endpoints to experiments using n=30 animals can be considered much more costly than applying them to experiments using n=5 animals, as application of surrogate endpoints necessitates monitoring on the level of individual animals.

Highly divergent views among the scientific community regarding the ability of fish to percept pain and suffering [39] might constitute another possible reason for the lack of information on surrogate endpoints in this field. It appears that there is also a traditionally higher tolerance towards mortality in fish research [17], probably because fish, due to their perceived differentness in morphology and behaviour, are less likely to be anthropomorphized by humans than terrestrial mammals.

Whatever might be the reasons, expansion of fish farming industry creates an increasing need to perform infection experiments in certain food-fish species [11, 26]. Intensive production, which generally involves husbandry of animals in high densities, together with increasing awareness towards bacterial antibiotic resistance, make vaccines and immune stimulants an important economical issue. For example, for market release and quality control of inactivated vaccines regulatory authorities generally demand *in vivo* testing, as there has been little progress in the development of replacement methods in fish [40]. To demonstrate efficacy of a vaccine, in general the occurence of lethality is required in the untreated control animals [11, 19, 26]. The obvious lack of information on surrogate endpoints in scientific literature and regulatory guidelines concerning fish, together with the high numbers of fish used for vaccine efficacy studies lead to the concern expressed by Cooper & Jennings (2008) that efficient surrogate endpoints are unlikely to be applied. The suspicion is unavoidable that this might be generally true for infection experiments on fish that require lethality as an endpoint. The use of the so called "moribund stage" as experimental endpoint in those experiments might be regularly claimed, but is regarded as questionable for multiple reasons, which to discuss are out of scope of this text (see [15] for further reading).

The aim of this study was identification and statistical validation of **potential death-predictors (PDP)** in form of visually perceptible clinical signs by video observation for two different infection experiments in rainbow trout *Oncorhynchus mykiss* (Walbaum). The rainbow trout presents an economically important food fish species worldwide and is commonly produced in aquaculture. The two bacterial pathogens used were *Aeromonas salmonicida* and *Yersinia ruckeri*. *A. salmonicida* elicits a disease commonly referred to as Furunculosis and *Y. ruckeri* presents the causative agent of a disease generally known as Enteric Redmouth Disease. Both diseases are considered economically important and are regularly encountered in commercial fish farms producing salmonid fish [32]. Furunculosis presents one of the vaccine monographs of the European Pharmacopoeia, in which there is a potential for a challenge assay to be used as a routine batch potency test[6] [11].

[4] e.g. several monographs for vaccine potency tests in the European Pharmacopoiea demand n=30 control animals

[5] one method tested in the scope of this study was tagging via visible implant elastomer (VIE) from Northwest Marine Technology, Inc.®. Unfortunately it did not warrant enough visibility under infrared illumination, which was necessary during the experiment.

[6] Inactivated vaccines are less reliable to cause an immune response than live vaccines and therefore

Because of the difficulties associated with handling of fish, it was decided to search exclusively for visually perceptible clinical signs. Video recording enables simultaneous evaluation of several groups of animals by a single observer. In principle this study carries out the first steps of the instructions given by Morton (2000) for identification of key clinical signs, which are very close observation and documentation during the course of disease, followed by retrospective validation. Beforehand typical clinical signs of Furunculosis as well as Enteric Redmouth Disease were listed from literature. Subjective reports from technical staff and researches, who were regularly involved in infection experiments performed at the Centre for Fish and Wildlife Health of the Vetsuisse Faculty Bern were gathered. It was considered important to take information about the pathogenesis of the respective diseases into account, as clinical signs of disease might vary between the two pathogens.

Different types of visually perceptible clinical signs were taken into consideration: **i) deviations from normal morphology**; in a number of diseases, organisms show changes of certain morphological features. This can be caused by pathophysiological mechanisms, which characterise the respective disease, or can be caused indirectly by behavioural changes that influence the appearance of an animal. The morphological alterations caused by a disease can be thereby specific or unspecific. Two commonly described unspecific morphological abnormalities that can be seen in rainbow trout suffering from bacterial infection are for example exophthalmia [7] and overall darkening of skin colour. A more pathogen-specific morphological abnormality is the formation of furuncle-like lesions in rainbow trout infected by *A. salmonicida*; **ii) deviations from normal behaviour**; typical but relatively unspecific behavioural changes that occur in a wide range of vertebrates are for example cessation of feeding or lethargy. Those behavioural deviations are designated as sickness behaviours [16, 21, 5]. Moving to a particular place in the tank (e.g. swimming near water surface) or to a particular position relative to other animals (e.g. segregation from the group) might also indicate deviation from normal behaviour; **iii) deviations from normal movement patterns**; A change of movement pattern might indicate neurological, motor and cardiovascular dysfunctions. For example, uncoordinated swimming with rotation around cranio-caudal body axis could be caused both by neurological dysfunctions (e.g. encephalitis, meningitis) or circulatory failure (e.g. cardiac insufficiency, multi-organ failure).

Because survival time was thought to influence the expression of clinical signs, different dosages were administered (similarly to an efficacy study) causing animals to demonstrate different time courses of disease. Vaccinated animals were included in order to record eventual signs of disease in this group, as it was assumed by the planners of this study that vaccinated animals might show different course of disease, provided disease occurs at all in those animals. Animals were housed in comparably small group sizes (n=10) per tank during the experiment. Tracking the progression of clinical signs in individual animals limited the number of animals inside the tank. Provision of structural enrichment inside the tank was restrained, because of possible interference with the visibility of the fish.

Any assessment done by a human observer is known to be vulnerable to error [44]. However, subjective evaluation of the health status of experimental animals can still be considered standard practice. In this study intra-observer reliability was assessed by re-evaluating video-sequences through the same observer, determining agreement between the first and the second observation. To facilitate reproducibility of results gained by a human observer, it was aimed to develop a systematic approach.

The author's tasks within this study were the following:

1. Designing and installing a technical system that would enable continuous video-recording of the fish inside the experimental tanks for several days.

2. Performing infection experiments, which were already planned and authorised by the responsible animal welfare agency.

3. Planning and performing video-observation and analysing the resulting data.

require testing for every vaccine batch.

[7] protruding eyes

Chapter 2

Materials and Methods

2.1 Technical set-up

Eight surveillance cameras (Model: IP8173H Mini-box Network Camera) from the company VIVOTEK® were used to continuously record the fish during the infection experiments. This model was selected i) because of the ability to record videos with high image quality up to 2048×1536 Pixel; ii) removable IR-cut filter for recording with visible light, as well as infrared light; iii) a CS-mount lens, which made manual control of focal length possible. It was originally planned to record in colour with visible light during the day, but because sensitivity of the camera in day-mode was too low, recording took place exclusively in "night-mode" with infrared light illumination. Infrared light was emitted by external spotlights (ABUS® TV6700) 24h/day. Each tank was equipped with two infrared-spotlights to establish high-quality recordings, as video resolution was highly dependent on light conditions. Spotlights were mounted over the tanks, the socket of the lamp being in approximately 37 cm distance from the water surface. Illumination with the same direction as the camera's axis (through the front glass) would have provided optimal visibility. However, this was not possible because of disturbing reflections from the aquarium glass. Cameras were mounted inside black plastic boxes (AUER® 60 × 40 × 42 cm) in front of the aquaria to avoid reflection of daylight against the glass. The videos recorded by the network cameras were processed by a HP®XW9400 Workstation with 2× AMD Dual Core CPUs with liquid cooling and 8 GB of RAM. The operational system was WINDOWS® Server 2008R2. The data was stored on four WD ® My Book external drives (4 TB each).

2.2 Conduction of infection experiments

2.2.1 Design of infection experiments

Four timely separated infection trials were performed with two different pathogens: two trials with *Aeromonas salmonicida* and two trials with *Yersinia ruckeri*.

1.	AsT1	(11.02.- 21.02.2016)	*Aeromonas salmonicida*	n=60
2.	AsT2	(23.02.-07.03.2016)	*Aeromonas salmonicida*	n=80
3.	YrT1	(05.04.- 15.04.2016)	*Yersinia ruckeri*	n=60
4.	YrT2	(20.04.- 01.05.2016)	*Yersinia ruckeri*	n=80

In AsT1/T2 fish were infected with *Aeromonas salmonicida*. In all trials ten fish were assigned to each tank. In AsT1 five different dosages were applied, one dosage being administered to each group of ten animals (overall number of animals infected n=50). There was one control group (overall number of animals that were not infected n=10). Each tank contained solely animals that had received the identical bacterial dosage. In AsT2, an identical dosage was given to four groups of each ten fish (overall number of animals infected n=40). There were four control groups (overall number of animals that were not infected n=40). In YrT1/T2 fish

Table 2.1: Groups and dosages - Overview

AsT1				AsT2			
Tank ID	Group	Target dose/fish (by injection[1])	Dilution factor of the stock solution (TSS)	Tank ID	Group	Target dose/fish (by injection[1])	Dilution factor of the stock solution (TSS)
T1	Inf.	50	1/100	T1	Inf.	100	0
T2	Inf.	100	1/50	T2	Inf.	100	0
T3	Inf.	500	1/10	T3	Inf.	100	0
T4	Inf.	1000	1/5	T4	Inf.	100	0
T5	Inf.	5000	0	T5	Contr.	0	-
T6	Contr.	0	-	T6	Contr.	0	-
				T7	Contr.	0	-
				T8	Contr.	0	-

YrT1				YrT2			
Tank ID	Group	Target dose/fish (by immersion[2])	Dilution factor of the stock solution (TSS)	Tank ID	Group	Target dose/fish (by immersion[2])	Dilution factor of the stock solution (TSS)
T1	Inf.	10^4	1/100	T1	Inf.	10^6	0
T2	Inf.	2.5×10^5	1/4	T2	Inf.	10^6	0
T3	Inf.	5×10^5	1/2	T3	Vacc.	10^6	0
T4	Inf.	7.5×10^5	1/1.3	T4	Vacc.	10^6	0
T5	Inf.	10^6	0	T5	Vacc.	10^6	0
T6	Contr.	0	-	T6	Vacc.	10^6	0
				T7	Contr.	0	-
				T8	Contr.	0	-

Inf. = Infected group ; Contr. = uninfected control group ; Vacc. = infected vaccinated group.
[1] Number of bacteria (CFU) per 50 μl of injection solution.
[2] Number of bacteria (CFU) per ml of immersion bath.

were infected with *Yersinia ruckeri*. In YrT1, five different dosages were administered to each group (overall number of animals infected n=50), with a single control group (overall number of animals that were not infected n=10). Again, each tank contained solely animals who had received the same bacterial dose. In YrT2, the identical dosage was given to two groups of each ten fish (overall number of animals infected n=20). There were two control groups (overall number of animals that were not infected=20) and four groups with vaccinated animals (overall number of infected, vaccinated animals n=40). For an overview and more detailed information about dosages, see Tab.2.1. Groups of fish were randomly assigned to the experimental tanks with the limitation that infected and control groups were not placed directly next to each other. Otherwise disinfection procedures during daily cleaning procedures would have been rendered too time consuming.

2.2.2 Animals and husbandry

Animals

Juvenile 0+ rainbow trout (*Oncorhynchus mykiss*) were obtained from two different commercial fish farms. Both farms produce animals for human consumption. Fish assigned to AsT1/T2 were raised in Switzerland (HOFER® strain). Animals were exclusively female. Fish assigned to YrT1/T2 were bred in France. Information about gender-composition is lacking. For both origins,

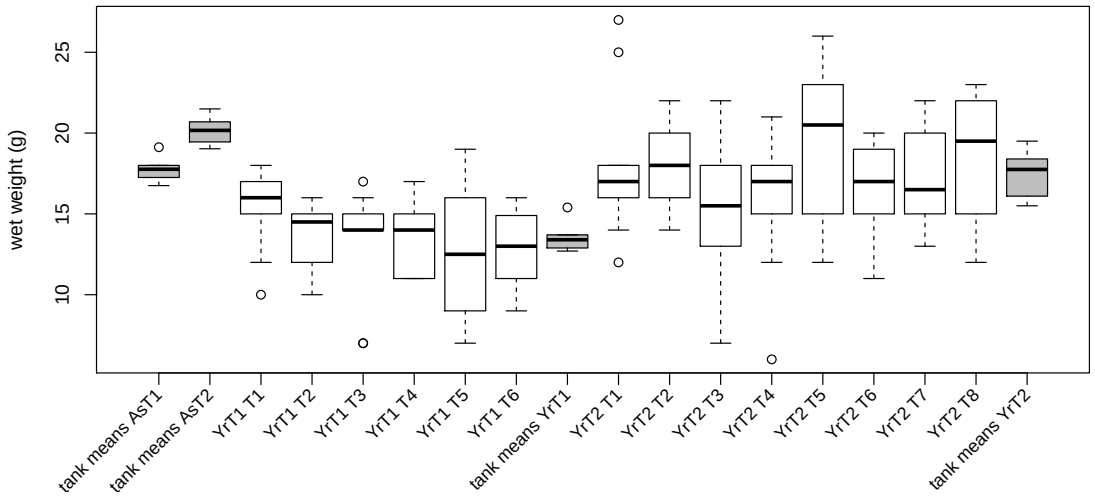

Figure 2.1: Distribution of body-weight (g) of fish at start of bacterial challenge. Grey boxes show distribution of tank means (for calculation method see Tab.2.2). White boxes show distribution of individual fish weight inside each tank (values only available for YrT1/T2).

the exact age of fish is unknown to the author. For data about the fish's body weight at beginning of each trial see Fig.2.1.

Husbandry before bacterial challenge

After arrival at the institute's fish-keeping-facility, animals were transferred to 57.5 × 58 × 37 cm [length × width × height] (volume = 130 l) glass aquaria. Fish destined for YrT1/T2 were once additionally transferred to smaller glass aquaria measuring 28 × 40 × 33 cm [length × width × height] (volume = 38 l) , because animals started to show increased territorial aggression in form of chasing and biting each others fins, which was assumed to be connected to the low density of animals in the tank after division in multiple smaller groups (vaccinated, sham-vaccinated animals and untreated groups). Tanks were illuminated by natural daylight falling through skylights and additional artificial illumination. Body weight was measured by group weighing of 10 randomly sampled animals without anaesthesia in a vessel containing fresh, aerated water with the whole procedure taking 1-2 minutes. Fish were fed commercial trout feed (HOKOVIT®). For further details about water temperature, amount of feed (% feed of body mass) and medical treatments see Fig.2.2. Fresh water supply was maintained by a flow-through system, consisting of Bern tap water with drink water quality. Each aquarium was equipped with one aeration stone. Faeces were removed daily at approximately 8:30 a.m. by siphoning. Developing biofilm was removed from the tank walls manually at least once per week.

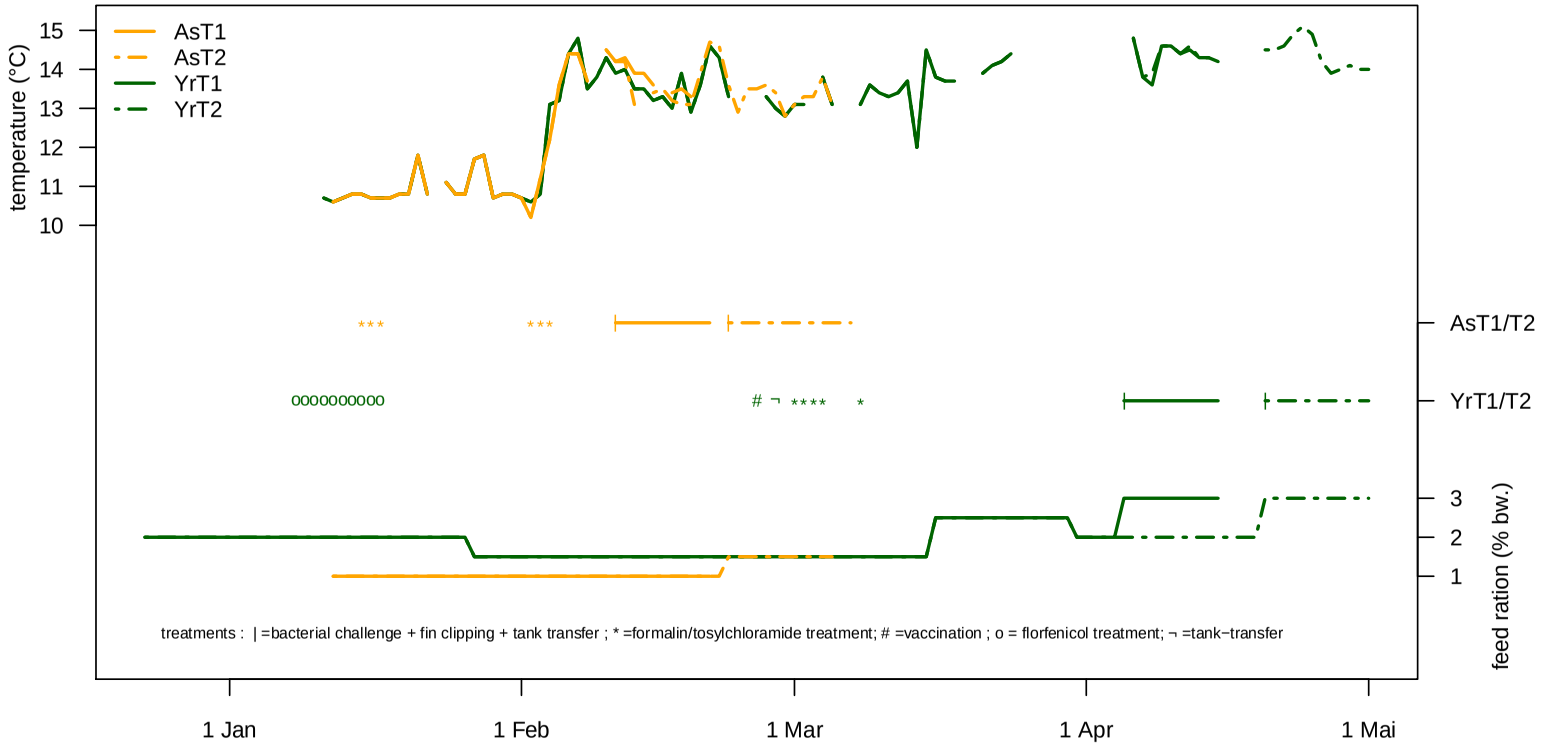

Figure 2.2: Husbandry conditions before and after experimental trials for AsT1/T2 and YrT1/T2; data about temperature is given in the upper third of the plot, the middle third depicts duration of each experimental trial plus medical and miscellaneous treatments. The bottom third depicts feeding rate (% of wet body weight) of fish designated to each trial.

Medical treatments before bacterial challenge

All fish received disinfection treatments in form of alternating formalin and tosylchloramide baths (for details about times of treatments see Fig.2.2) as clinical signs (opaque patches of skin, erosion of dorsal fin) and microscopic examination of wet mounts of skin and gills supported the suspicion of presence of skin parasites *Gyrodactylus spp.* and fish pathogenic bacteria *Flavobacteria spp.*. Because of an outbreak of systemic Flavobacteriosis, fish provided for YrT1/T2 received an oral antibiotic treatment (SHOTAFLOR®; active ingredient: Florfenicol; dose: 15 mg/kg for 10 days). Treatment ended 79(YrT1)/94(YrT2) days before bacterial challenge, which corresponds to approximately 1046(YrT1)/1256(YrT2) day degrees under the given temperature regime between end of antibiotic treatment and bacterial challenge.

Experimental tanks and husbandry during bacterial challenge

After (sham-)infection and fin-clipping, fish were immediately transferred to the experimental tanks. Those consisted of 28 × 40 × 33 cm [length × width × height] (volume = 38 l) aquaria, which were further divided into two physically separated compartments by perforated partition walls made of plastic. The perforated partition walls enabled water to circulate freely through the whole tank while fish were prevented from changing compartments. Ten fish per tank were transferred into the front compartment measuring 28 × 22 × 25 cm [length × width × height] (volume = 15 l). Fish were fed twice daily at 9:00 a.m. and 4:00 p.m. with HOKOVIT® commercial trout food. For information regarding amount of feed during the trials see Fig.2.2 and/or Tab.2.2. Water supply consisted of tap water. Water flow had to be regulated manually without a flowmeter system. Flow rates were randomly measured before start of experiment and were between 1.6 and 2.4 l/min (96 × 144 l/hour), meaning a exchange rate of the aquaria's water between 2.5 to 3.8 times per hour. Available data on water temperature is depicted in Fig. 2.2. Fish were submitted to an artificial dark/light regime. From 7:00 a.m. to 7:00 p.m. they received indirect artificial light from a warm-white 2.5 Watt LED light bulb (one bulb per two aquaria). The light intensity during this time was very low. Several attempts to log light intensity with a luxmeter (HOBO Pendant® temperature/light logger) failed, because the device could not detect any light despite of a detection range 0 - 323,000 lumens/m^2. In AsT1/T2, each pair of aquaria was separated by a transparent glass partition wall, which made visual contact between two experimental groups possible. In YrT1/T2 the transparent wall was made opaque, so the fish of two groups were no longer able to see each other through the glass. In AsT2 a partition wall was installed in the upper half of each experimental tank, in order to provide visual shielding and coverage for the animals. This was done in an attempt to attenuate aggressive behaviour, which has been observed in the foregoing trial AsT1. In the trials YrT1/T2 another attempt was made to alleviate intra-specific-aggression among the animals, by providing a current inside the experimental tanks, as experiments with Arctic charr *(Salvelinus alpinus)* have shown that provision of a moderate current can reduce aggressive behaviour among the salmonid fish [4]. The current was created by installation of an aquarium pump (EDEN® 109, maximum flow = 500 l/h, maximal velocity = 0.80 m/sec). The pump was adjusted to the maximal flow rate. For an overview about differences between the trials in pathogen, time, design of experimental tanks, origin of animals see Tab.2.2.

Marking of fish

Fish were individually marked via fin-clipping together with infection procedure. The day before individual marking and infection the animals were deprived of feed. Fish were sampled out of the holding-tank in groups of 10 animals and transferred into a tank containing aerated water with 50 mg/l tricaine methanesulfonate (MS-222) and bicarbonate buffer. As soon as the animals showed loss of equilibrium, cessation of caudal fin movement and no reaction to handling, they were individually transferred into a vessel that was specifically designed by the author for the purpose of fin-clipping. While being inside this vessel, the fish's head and trunk were kept under water, while

Table 2.2: Differing factors between trials - Overview

Trial	**AsT1**	**AsT2**	**YrT1**	**YrT2**
Date of conduction	11.02.-21.02.2016	23.02.-07.03.2016	05.04.-15.04.2016	20.04.-01.05.2016
Pathogen	*Aeromonas salmonicida*	*Aeromonas salmonicida*	*Yersinia ruckeri*	*Yersinia ruckeri*
Infection route	Intraperitoneal injection	Intraperitoneal injection	Bath immersion	Bath immersion
Mean weight of fish (g) [1]	17.77	20.14	15.4	18.1
Mean density (kg/m^3) in experimental tank (water volume that was accessible to fish)	11.8	13.42	10.2	12.06
Mean density (kg/m^3) in experimental tank (water volume that was accessible to the fish + non-accessible water volume)	4.67	5.3	4.05	4.7
Feed ration 2 weeks before trial (% of average body weight)	1.00%	1.00%	2-2.5%	2.00%
Feeding ration during trial (% of average body weight)	1.00%	1.50%	3.00%	3.00%
Transparent glass wall between two aquarium units	Yes	Yes	No	No
Room divider	No	Yes	No	No
Pump generating a current	No	No	Yes	Yes
Origin of fish	Commercial fish farm A[2]	Commercial fish farm A[2]	Commercial fish farm B[3]	Commercial fish farm B[3]

[1] Group of fish (n=10) were weighted for each tank. The given mean weight per trial was therefore calculated from the mean of all tank means.
[2] Commercial fish farm A presents the Swiss fish farm. Fish from AsT1/T2 originate from the same batch.
[3] Commercial fish farm B presents the French fish farm. Fish from YrT1/T2 originate from the same batch.

caudal, anal, abdominal and dorsal fin were accessible for clipping. The vessel was constructed out of polystyrene and its surface was covered with a layer of silicon (so called "kitchen-silicon", manufactured for the food-sector) to protect the fish's skin from potential damage. Water inside the vessel contained MS-222 (50 mg/l) plus bicarbonate buffer and was exchanged after every 10^{th} animal. The aim was to maintain respiratory activity during the procedure and minimize metabolic stress by oxygen-deficiency. For fin-clipping a cuticle-remover-forceps was used, allowing clean and quick (10 - 15 seconds per fish) removal of triangular pieces ($\approx$ 3-5 mm edge length) of the fin. Each fish had two clippings to avoid eventual confounding of the amount of injury per animal (for the fin-clipping scheme see Fig.B.3 in Appx.B). During all handling procedures the executing persons wore latex gloves to protect the animal's skin.

Preparation of bacterial solution

For bacterial challenge of fish in AsT1/AsT2 an archived, virulent strain of *A. salmonicida* (isolate JF5055) was used. Bacteria were grown on Tryptycase soy agar at 15 °C. The stock solution (TSS) was produced by suspension, measuring 1 McFarland Turbidity standard ($\approx 3 \times 10^8$ CFU/ml) by bioMérieux® DENSIMAT. The stock solution was repeatedly drawn up into a syringe and ejected again through a 21-gauge needle for 5 minutes to achieve a homogeneous suspension without agglomerations of bacteria to decrease variation of bacterial doses between fish. From this stock solution, several dilution series (using sterile PBS) were made and subsequently used as injection solution. For *Yersinia ruckeri* a bacterial strain (Biotype 2) isolated from a natural outbreak was used for infection. Bacteria were grown on Tryptycase soy agar at 15 °C. A stock solution was prepared by suspension and consequently three 1:3 dilutions were produced. For 5 minutes the stock solution was repeatedly drawn up into a syringe and ejected again through a 21-gauge needle to achieve a homogeneous suspension without agglomerations of bacteria. McFarland standards of the three dilutions were measured and the theoretical concentration of the stock solution was calculated from those three measurements. Then the amount of stock solution that was needed to achieve the desired bacterial concentration for bath immersion was calculated. This protocol was chosen because of the necessity to produce comparably large amounts of highly concentrated bacterial solutions. For details about the intended dosages for all trials see Tab.2.1

Infection procedure in AsT1/T2

In experiment AsT1/T2 animals received an intraperitoneal injection of 50 μl with either bacterial solution or sterile PBS directly after fin clipping. Subsequently, fish were transferred to another tank containing anaesthetic (50 mg/l MS-222 plus bicarbonate buffer) until all ten fish assigned to one experimental tank were marked and injected. In this way all fish were subjected to anaesthesia for the same amount of time, which was ten to twelve minutes. In the end, fish were weighed together in groups of ten, in a water filled vessel and transferred to the experimental tank.

Infection procedure in YrT1

In experiment YrT1 fish were infected via bath-immersion before fin-clipping took place. Fish were randomly assigned to different tanks (10 fish per tank) containing fresh, aerated tap water from the same source as the husbandry tanks. Bacterial solutions (except for control groups) were added to the tanks and fish were left for 60 minutes. Afterwards, water was exchanged with fresh water in all tanks and fish were put batch-wise into narcosis (50 mg/l MS-222 plus bicarbonate buffer), fin-clipped, individually weighted, measured (fork-length) and batch-wise transferred into their experimental tanks.

Vaccination, sham-vaccination and infection procedure in YrT2

Forty fish were vaccinated 54 days ($\approx$ 8 weeks) prior to bacterial challenge against *Y. ruckeri* biotype 2 by bath immersion according to the manufacturer's instruction (Aquavac ® RELERA).

Table 2.3: Examinations performed in each trial - Overview

Trial	AsT1	AsT2	YrT1/T2
Individual weight (initial and final)	-	-	+
Fork length (see Fig.3.4) (initial and final)	-	final fork length	+
Bacteriological examination head kidney (re-isolation)	-	+	+
Gross pathological examination	-	+	+

The control groups were submitted to the same handling procedures, except that their tank water did not contain any vaccine. Bacterial infection and fin-clipping, weighing and length measurement were performed identical to YrT1.

Re-isolation and gross pathological examination

In experiment AsT2 and YrT1/T2 bacterial examination of the head kidney of every individual fish was performed, either after death or after euthanasia at the end of the experimental period. This was done by streaking head kidney material, which has been taken with a sterile platinum loop, on blood-agar-plates used for routine diagnostics. Agar-plates were checked for presence or absence of bacterial growth during the following three days. In case of bacterial growth, it was visually evaluated for homogeneity and typical morphology of *A. salmonicida* and *Y. ruckeri* respectively. In cases of doubt, bacterial identification via MALDI-TOF mass spectrometry was performed. Additionally, gross pathological examination was performed (except in AsT1) and pathological findings were noted. See Tab.2.3 for an overview.

2.3 Video-observation and statistical analysis

2.3.1 Data collection

Video observation was done exclusively by one person (the author). For displaying the videos, the free video management software ST7501 Version 1.11 by VIVOTEK® was used. Recorded videos were first screened to establish the exact time of death of each individual animal that succumbed to infection during trial. The process of screening did not follow a fixed scheme, but had more exploratory character. Approximate time of death of each animal was known from experimental documentation and served as orientation. During screening, **visually perceptible traits (VPT)** that might indicate illness were noted. Fish showing no respiratory activity in form of opercular movement and no motion of fins where considered to be dead. After time of death was determined for all animals, it was specified which of the VPTs collected during the exploratory screening-process should be assessed systematically via video-observation (for complete list see Tab.3.2 in Chap.3). Those VPTs were documented for every individual animal according to the following scheme: 72, 68, 64, 60, 56, 52, 48, 46, 44, 42, 40, 38, 36, 34, 32, 30, 28, 26, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5.5, 5, 4.5, 4, 3.5, 3, 2.5, 2, 1.5, 1, 0.5 hours before death (=48 time points for every fish). This was done solely in animals that died during the trial (= nonsurvivors) and for which re-isolation of the respective bacterial agent was successful.

Four VPTs (Anorexia; DVax = 90°; DVax = 180°; DVax = 0-180°, for definition see Tab.3.2 in Chap.3) were assessed also in the animals surviving the trial, whereby a different time scheme was adopted. While anorexia was assessed twice daily at 9:00 a.m. and 4:00 p.m., DVax was assessed in survivors by screening the whole experimental period every three hours (e.g. 00:30

a.m., 03:30 a.m.) for 30 seconds. Anorexia was assessed for survivors in all four trials. The first two feedings (18 hours and 25 hours after bacterial challenge) were excluded from analysis of anorexia because feeding behaviour at this time was considered to be impaired by the lack of acclimation. DVax was only assessed in survivors of AsT1 due to temporal constriction. If the respective pathogen was re-isolated from a survivors kidney, the animal was excluded from the analysis, as it cannot be said if these animals were on their way to recovery or on the way to succumb to infection. Those animals therefore presented unclear cases and could neither be allocated to the survivors, nor to the nonsurvivors.

2.3.2 Graphical and numerical analysis of data

All visualisation and statistical evaluation of data was done using statistical software R [34]. Data gained by video-observation were plotted as strip-charts for first graphical visualisation. Certain VPTs and combinations of VPTs were chosen as potential death predictors (PDPs). The selected PDPs were subsequently analysed by graphical visualisation and calculation of statistical key figures, described in Tab.2.4. After evaluation of strip-charts and statistical key figures separately for each of the four trials, the author found that general patterns (e.g. clinical signs, chronological sequence of the respective PDPs in individual animals as well as proportions of nonsurvivors showing those PDPs) were not found to be obviously different between the first and the second trial (AsT1 and AsT2). The same was found for the third and fourth trial (YrT1 and YrT2). In contrast, there seemed to be differences in symptoms, chronological patterns and proportions between AsT1/T2 and YrT1/T2. Values of individual animals from AsT1 and AsT2 were therefore analysed as one unit and the same was done for YrT1 and YrT2.

Additional statistical analysis with regard to a possible correlation between social stress and complete anorexia

It was suspected by the author, that apart from the sickness induced anorexia, social stress presented an additional anorexigenic factor during this experiment. As lesions in form of injuries were considered to correlate with social stress perceived by an animal during this trial, the author believed that the proportion of fish displaying medium to severe damage should be higher in anorectic compared to non-anorectic, control- and vaccinated animals. This was considered not to be true for the survivors of infection, as it was assumed that illness, social stress, as well as reciprocal interactions between illness and social stress were likely to have influenced the occurrence of complete anorexia (see chapter 4). Unfortunately, external lesions were not assessed in AsT1, therefore those fish could be not included in the analysis. Animals from YrT1 and YrT2 were analysed as a single group. For numerical analysis Fisher's exact test for count data was performed (details see Fig.3.16 and 3.18).

Additional statistical analysis with regard to potential correlation of survival time and forecast time in complete anorexia

From graphical analysis, the impression emerged that fish dying late during the course of experiment showed a proportionally longer duration of complete anorexia, compared to fast dying animals. For analysing a potential correlation of survival time and forecast time of the PDP cAnor, least-squares linear-regression was performed (independent variable= t_{surv}, dependent variable= t_{Foc} / t_{surv}).

Intra-observer reliability

To evaluate the reliability of results gained by video-observation, several PDPs were tested for intra-observer reliability. This was not only done because of the general subjectivity that's unavoidable in assessment done by an human observer, but found to be especially important with regard to the fact, that technically and timely constraints made it impossible to evaluate all the

Table 2.4: Key Statistics calculated for each Potential Death Predictor (PDP)

Denotation	Abbr.	Definition	Explanation	Remarks
Sensitivity	Sens	Sens =($n_{PDPdead}/n_{dead}$)×100 $n_{PDPdead}$= number of animals that didn't survive bacterial infection and that were at least once observed showing the respective PDP n_{dead} = number of animals that didn't survive bacterial infection. For each PDP Sens(AsT1/T2) and Sens(YrT1/T2) were calculated.	Percentage of nonsurvivors correctly identified by presence of the respective PDP.	-
Specificity	Spec	Spec = ($n_{noPDPsurv}$ / n_{surv})×100 $n_{noPDPsurv}$ = number of animals that survived bacterial infection and that were never observed showing the respective PDP n_{surv} = number of animals that survived bacterial infection	Percentage of surviving animals correctly identified by the absence of the respective PDP.	Specificity could only be determined for few PDPs (DLR, DLR+sevPi, cAnor) , as data collection turned out to be more time consuming than expected.
Survival time	t_{surv}	$t_{surv} = t_{inf} - t_{death}$ t_{inf} = time of bacterial infection t_{death} = time of death	t_{surv} gives the duration between time of bacterial infection and time of death of an individual animal.	-
Time to first/second Observation	t_{Obs}	$t_{Obs} = t_{inf} - t_{PDP}$ t_{inf} = time of bacterial infection t_{PDP}= time of first/second observation of the respective PDP in an individual fish. Following statistics were calculated: 25%, 50% and 75% quantiles, median absolute deviation (MAD) of t_{Obs}.	t_{Obs} gives the interval between time of bacterial infection and time of first or second observation of respective PDP. This value was calculated for each individual animal showing the respective PDP.	Precision of t_{Obs} is largely dependent on the density of observation points. Density of observations increased while animal was approaching death (see section Material and Methods about video observation), meaning that t_{Obs} is the more accurate, the closer to death the first/second observation of a respective PDP emerged.
Forecast time	t_{Foc}	$t_{Foc} = t_{surv}$ - t_{Obs} t_{surv}= survival time (see above), t_{Obs} = time to first/second observation (see above) t_{Foc} was calculated for each nonsurvivor that showed the respective PDP. Following statistics were calculated: 25%, 50% and 75% quantiles , median absolute deviation (MAD) of t_{Foc}.	t_{Foc} gives the interval between time of first or second observation of the respective PDP and time of death. For effectively reducing the time that an animal spends inside an experiment, the question how well in advance death can be predicted is of crucial importance.	- Usually time of first observation was chosen to calculate t_{Foc}. For some PDPs (Abd, MLBo), which showed an intermittent presence with increasing consistency towards death, it was decided to take the second point of observation. - Precision of t_{Foc} is largely dependent on the density of observation points. Density of observations increased while animal was approaching death (see Chap.2 about video observation), meaning that t_{Foc} is the more accurate, the closer t_{PDP} is to t_{death}.
Percentage of non available observations	ObNA%	n_{ObNA} / n_{totOb} = ObNA%. n_{ObNA} = number of observations where presence or absence of the PDP could not be determined. n_{totOb} = total number of observations per fish (=48) ObNA% was calculated for each nonsurvivor that showed the respective PDP. Following statistics were calculated: 25%, 50% and 75% quantiles , median absolute deviation (MAD) of ObNA%.	- A PDP with high ObNA% indicates that the PDP of interest was frequently not accessible, e.g. the animal was not in a suitable position relative to camera or was partly hidden by another fish.	For anorexia, the calculation of ObNA% was not possible, as due to variation in survival time, the total number of observations differed strongly among individual fish. For the remaining PDP the number of observations in almost all fish was n= 48 (minus 1-3 observations in 5 fish, which was considered negligible). - For stiffened locomotion (SLoc) ObNa% is considered of limited reliability.

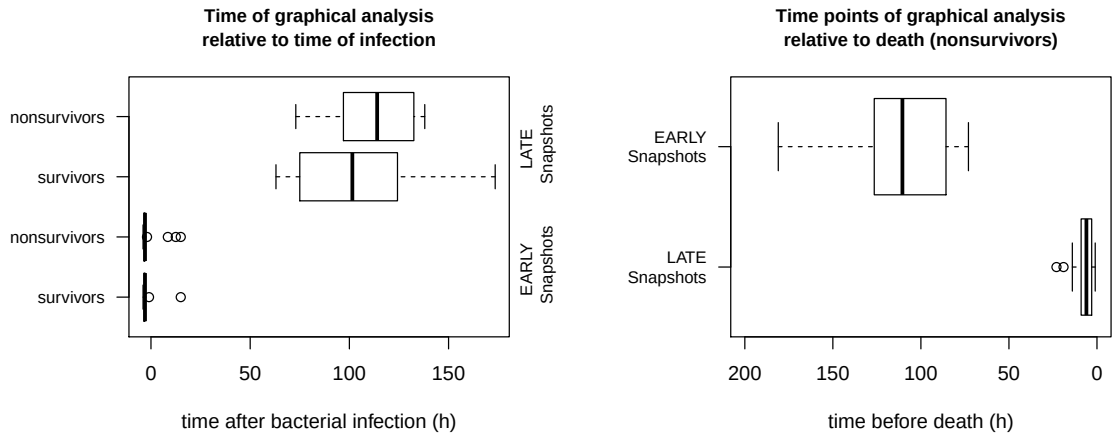

Figure 2.3: Timing of snapshots taken for graphical analysis of abdominal and dorsal curvature relative to time of infection (left) and time of death (right).

video material in a blinded fashion. During video watching, the observer was always aware of the identity of each fish and therefore of its status as surviving or nonsurviving. Furthermore time and date of the respective section of video was known to the observer, so he knew about the amount of lifespan an animal still had ahead of itself. This awareness raises the probability of subconscious bias of judgement by the observer. Therefore it was tested if the same results could be obtained by rescoring through the same observer. For every PDP (except severe perpendicular instability) 48 points in time, which have been evaluated via video-observation, were selected with help of a random sampling function in R statistical software. Half of points in time (=24) showed animals, which demonstrated the respective PDP according to the first evaluation by the author. The other half showed animals, which did *not* demonstrate the respective PDP according to the first observation by the author. Video clips lasting 20 seconds were extracted from the main video, presenting the sequence that was watched during the first observation. For each PDP the videos were put in a folder and subsequently **blinded** by renaming and resorting. The video-clips were then evaluated a second time by the same observer for the presence or absence of the respective PDP. Unlike for the first observation round, identity and remaining lifespan of the fish were not known to the observer at the second observation round. In the evaluation of some PDPs, a number of video-clips had to be removed from the analysis, because the fish of interest was not identifiable during the second observation. If a fish was not identifiable during the first observation round, it was possible to scroll the video either forwards or backwards until the fish was shown in a more favourable position to be identified. This was not possible in the second observation round, as the video-clips were 20 seconds extractions from the original video recording. From the resulting data, Cohens-Kappa was calculated to enable comparison between different PDPs.

Graphical analysis of dorsal and abdominal curvature

The shape of dorsal and abdominal curvature of fish were analysed from snapshots, which were extracted from video made during trial AsT1. Snapshots of nonsurviving fish (n = 27) were taken as well as of infected but surviving (n = 17) fish. Two snapshots of each fish were made: one snapshot directly after transfer to the experimental tank (= “early” measurement of the curvature) and a second snapshot few hours before death of the animal (= “late” measurement of the curvature). In case of the surviving animal the second snapshot was taken at a point in time that corresponded to the time of death of nonsurviving tank-mates, which had received the same dose. Distribution of points in time, when the Snapshots were taken are shown in Fig.2.3.

Snapshots were taken only, if the fish was positioned in the middle third of the tank height and displayed himself laterally, with his body parallel to the front glass. Furthermore, snapshots were only taken if the fish’s cranio-caudal axis was fairly straight. The snapshots were analysed with help of a software written in R. The cranial base of the abdominal fins, the cranial base

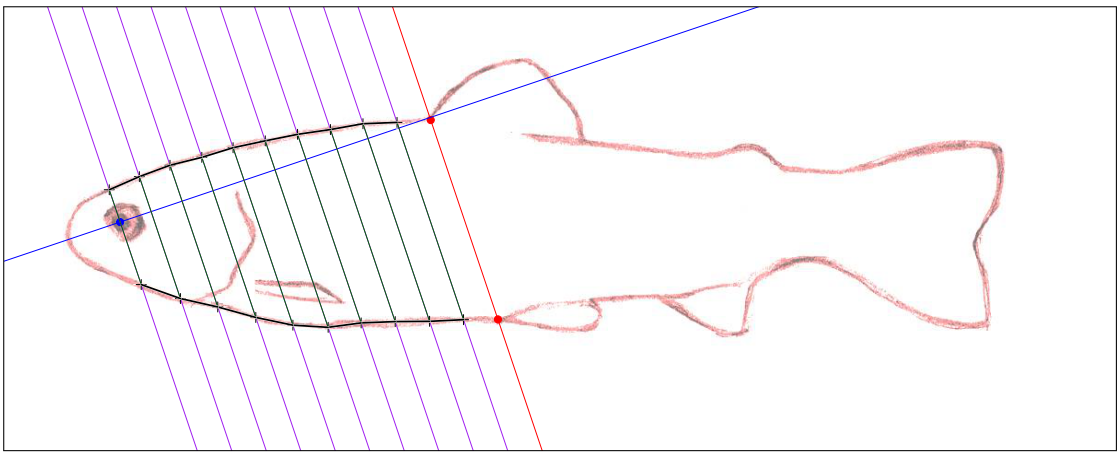

Figure 2.4: Graphical user interface of the software written in R showing the points of measurement. Ten coordinates each were determined for graphical analysis of abdominal and dorsal curvature.

of the dorsal fin and the eye were marked as fix-points by mouse-click. A first line was drawn between the cranial base of the abdominal fin and the cranial base of the dorsal fin. A second line, perpendicular to the first line was drawn through the eye of the fish. Between the crossing of the two lines and the eye, the second line was divided in ten intervals, and ten lines, parallel to the first line were drawn. By mouse-click, the intersections of these lines with the abdominal and dorsal outline of the fish were marked (see Fig.2.4). In this way, it was aimed to obtain coordinates, which would be comparable between animals, independent from their actual size or distance from the camera. Analysis was done by group visualisation of abdominal and dorsal curvatures. Dorsal as well as abdominal curvatures were plotted in four groups:

i early curvature-measurements of nonsurvivors,

ii late curvature-measurements of nonsurvivors,

iii early curvature-measurements of survivors,

iv late curvature-measurements of survivors.

As dorsal and abdominal curvatures have been captured by ten coordinates each, the distance between the 1st and the 10th coordinate as well as the position in the coordinate system were standardised for all fish, to allow comparison while keeping distortion to a minimum. One group contains n fish. The ith point of the jth fish is written $P_{\mathrm{ij}} = (x_{\mathrm{ij}}, y_{\mathrm{ij}})$. To visualise the distribution of the curvatures among this group, quantile curvatures are displayed. Given a percentile α, say 25%, 50% and 75%, each point of the quantile curvature is computed as follow:

$$P_{\mathrm{i},\alpha} = (\mathrm{Q}_\alpha(x_{\mathrm{i}1}, \ldots, x_{\mathrm{i}N}), \mathrm{Q}_\alpha(y_{\mathrm{i}1}, \ldots, y_{\mathrm{i}N}))\,,$$

with N the size of the considered group, and Q_α the quantile function of probability α.

Graphical evaluation of spatial distribution

During video-observation, it was found that fish seemed to show particular distribution patterns inside the tank. Some tanks showed high similarity in spatial distribution pattern, while others were quite distinct. This was often found to interfere with the aim to identify certain PDPs (e.g. settling motionless on the tank bottom). There also seemed to be a difference in spatial distribution between day and night. Subsequently an attempt was made to objectively assess the spatial distribution of fish inside a selection of tanks. It aimed to quantify the amount of movement recorded by the videos, to create a “heatmap” showing the distribution of movement

on the two-dimensional projection of the tank. Movement detection was achieved by quantifying changes in the grey-levels of pixels from a regular sequence of pictures extracted from the video (one picture per second). More precisely, the movement quantification for each pixel is:

$$M = (\ell_1 - \ell_2)^2 + (\ell_2 - \ell_3)^2 + \ldots + (\ell_{\mathrm{i}} - \ell_{\mathrm{i}+1})^2 + \ldots + (\ell_{N_{\mathrm{Fr}}-1} - \ell_{N_{\mathrm{Fr}}})^2$$

with ℓ_{i} is the the grey level at time i (a number between 0 and 1) and N_{Fr} is the number of frame in a period of 12 hours. The overall result is similar to a long time exposure in photography, but based on movement rather than brightness.

The author chose the 6^{th} day after beginning of the infection experiment for a video sequence ranging from 7:30 a.m. to 7:30 p.m. A video sequence of the following night was also extracted (from 7:30 p.m. to 7:30 a.m). All tanks contained control or vaccinated groups except two tanks. See the heatmap in Fig.3.22 and Fig.3.23 for the group designation. Feeding time were excluded from analysis. Tanks contained between eight and ten animals.

Chapter 3

Results

For the sake of clarity, this chapter is divided into two sections. Inside the first section, outcomes of the infection experiments are reported. The second section contains results gained by video observation.

3.1 Infection Experiments

3.1.1 Survival times

Survival times of fish that died in the course of AsT1 and AsT2 range from 70-215 h (≈3-9 days) and follow a right-skewed distribution, meaning that fish with short survival time are over-represented, with 50Q (t_{surv}) = 112.5 h (≈4.6 days). Survival times in YrT1 and YrT2 have a range of 97-190 h (≈4-8 days) and is slightly less right skewed, with 50Q (t_{surv}) = 135 h (≈5.6 days). Mortality rates and survival times of each tank are given in Fig.3.1.

3.1.2 Gross pathology

Lesions assumed to be caused by bacterial infection

AsT2: Main gross pathological findings in the fish having received an intraperitoneal injection of *A. salmonicida* were a reddened and rough parietal peritoneum, autolytic and opened body cavity, focal reddening of the skin on the ventral abdomen, a round and enlarged spleen, red coloured intraabdominal fluid, black coloured intestinal segments and a reddened injection canal (for proportions see Fig.3.2). A photo of a dissected fish that died during the trials and was re-isolated positive for *A. salmonicida* is given in Fig.B.4, Appx.B. None of these lesions were found in surviving animals that were tested negative for the presence of *A. salmonicida*.
YrT1/T2: Main gross pathological findings in the fish having received a bath-immersion with *Y. ruckeri* were pale gills, a rounded, swollen spleen, a stomach filled with water, a reddened swim-bladder, a pale liver, reddened skin in the head-area and oral cavity, pink coloured perivisceral fat, reddened parietal peritoneum in the caudal abdomen close to the spleen, pinhead sized dark-red discolorations in liver and periviszeral fat (for proportions see Fig.3.2). None of these lesions were found in surviving animals that were tested negative for the presence of *Y. ruckeri*. Five out of fourteen dead fish in the state of *rigor mortis* demonstrated flared opercula. Photos of fish that died during the trials and were re-isolated positive for *Y. ruckeri* are given in Fig.B.5, Appx.B.

Lesions not assumed to be caused by bacterial infection

The lesions found could be divided into two distinct groups: i) cranial lesions and ii) caudal lesions. Lesions were each categorized into three different degrees of severity according to the criteria given in Tab.3.1. Results are given in Fig.3.3. Cranial lesions were mainly observed in AsT2 (fish of AsT1 were not subjected to a gross pathological examination), while caudal lesions

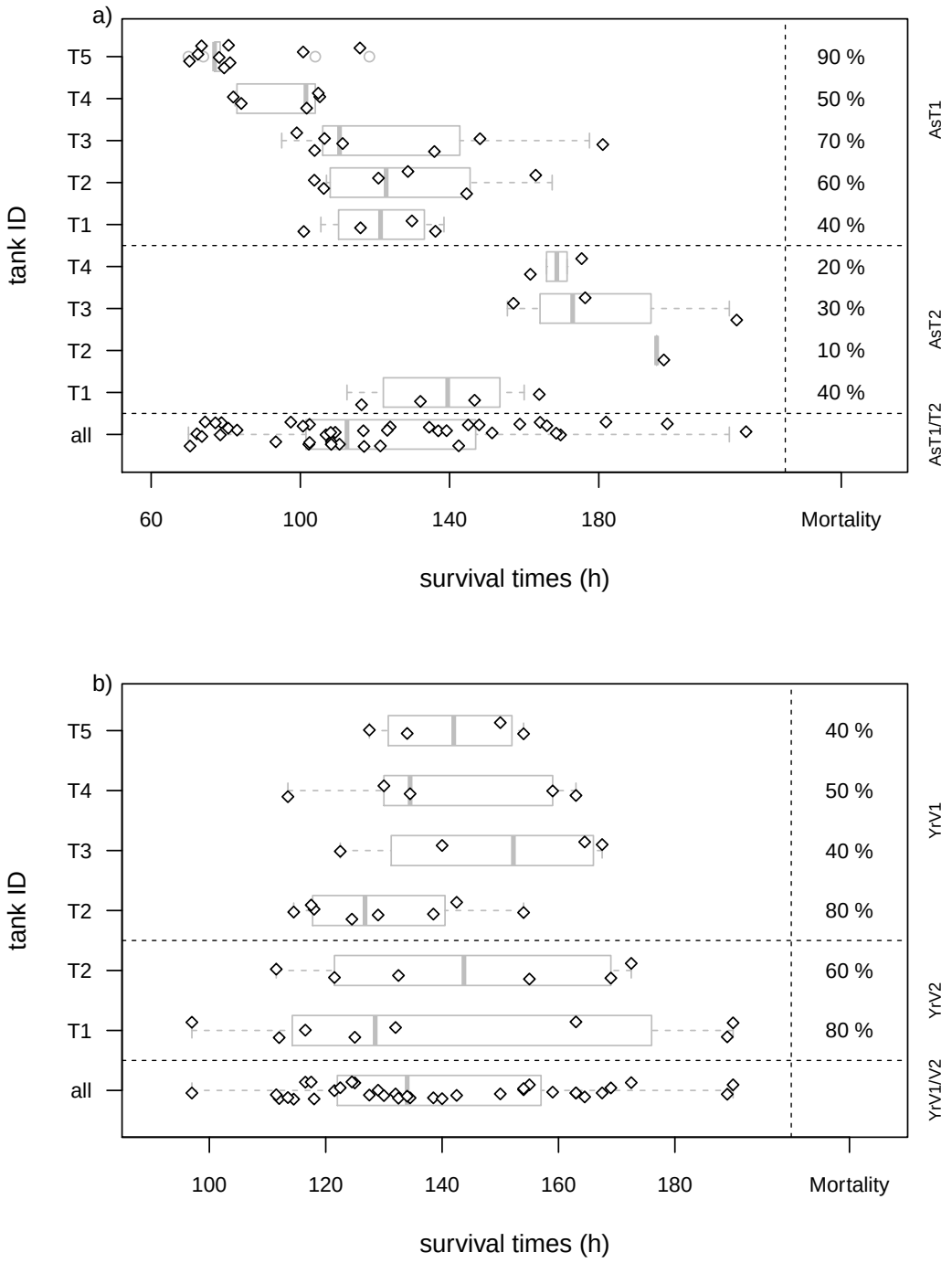

Figure 3.1: Distribution of survival times (t_{surv}) among each tank plus **Mortality rates (%)**; a) AsT1/T2 and b) YrT1/T2

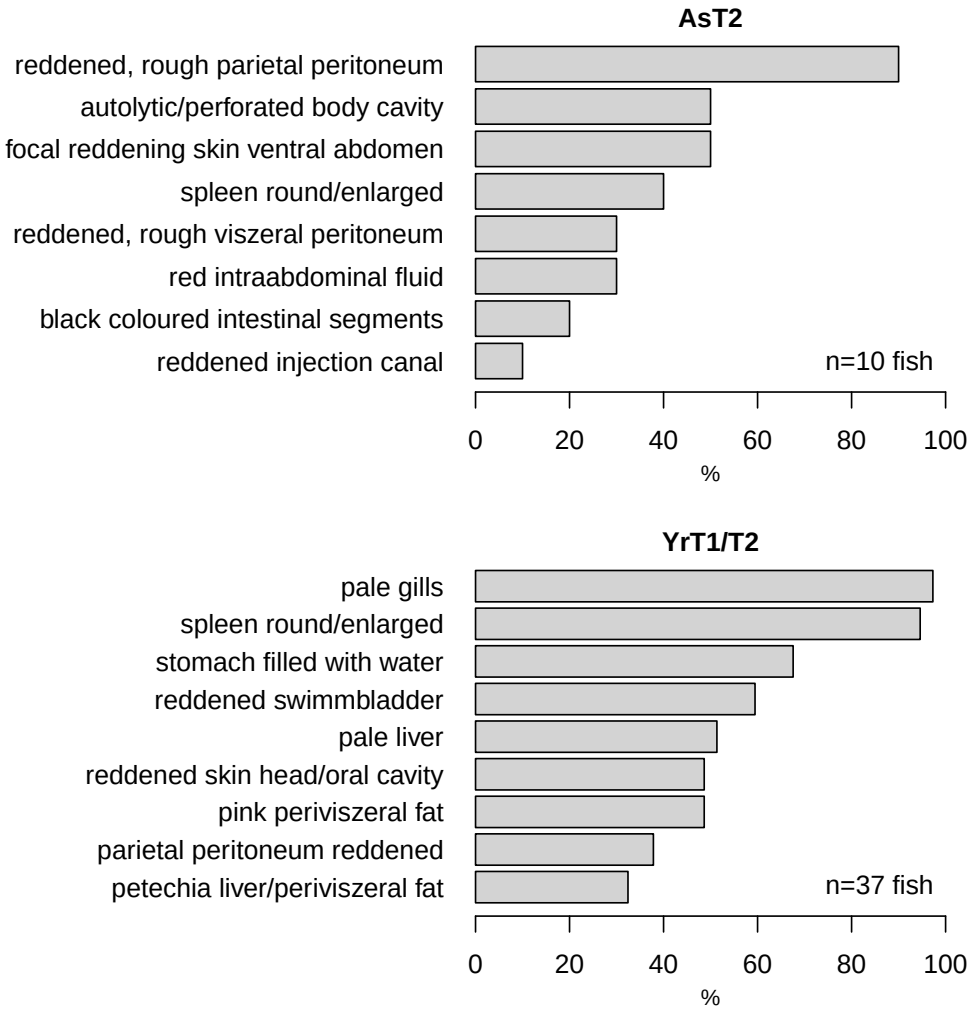

Figure 3.2: Infection-related lesions - Main findings of gross pathology in AsT2 (upper plot) and YrT1/T2 (lower plot). All lesions listed were assumed to be caused by bacterial infection of either *A. salmonicida* or *Y. ruckeri*.

Table 3.1: Criteria for categorisation of non-infection related lesions in AsT1 and YrT1/T2

Abbr.	Designation	Definition
Sn0	No snout lesion	Skin covering the tip of upper and/or lower jaw shows homogeneous colouration and no irregularity in surface structure.
Sn1	Snout lesion degree 1	Skin covering the tip of upper and/or lower jaw shows local whitish/yellowish colouration ($\approx$ 1-2 mm $\varnothing$) without irregularity in surface structure (perceivable by the bare eye).
Sn2	Snout lesion degree 2	Skin covering the tip of upper and/or lower jaw shows local whitish/yellowish colouration (>2 mm $\varnothing$) with clear irregularity in surface structure in form of local atrophy.
Sn3	Snout lesion degree 3	Skin covering the tip of upper and/or lower jaw is locally missing, revealing underlying tissue with pink to red colouration, exposing internal bony and cartilaginous structures of oral and/or nasal cavity.

Abbr.	Designation	Definition
FD0	No damage on caudal fin.	Fin rays and skin localised between fin rays are intact.
FD1	Caudal fin damage degree 1	Fin rays are intact, skin localised between fin rays is missing between two rays forming one or two deep slits inside the caudal fin or skin localised between fin rays is eroded until $\approx$ 90% of the length of the fin rays.
FD2	Caudal fin damage degree 2	Fin rays are intact , skin localised between fin rays is missing between two rays forming more than two deep slits inside the caudal fin skin localised between fin rays is eroded until $\approx$ 70% the of the length of the fin rays.
FD3	Caudal fin damage degree 3	Bony rays of the fin have been partially eroded and/or the skin localised between the fin rays is missing until the base of the fin. Local ulcerations, epidermal thickening and presence of blood vessels can be seen.

predominated in YrT1/T2. In Fig.B.6 and Fig.B.7 several photos of lesions that occurred during the trials are provided.

Evaluation of wound healing progress in clipped fins:

For AsT2 and YrT1/T2 special attention was paid to whether or not the animals showed impairment of wound healing in the area of fin clippings. In fish where anal, caudal and abdominal fins were *not* abraded (which was the case for most of the fish in AsT2) there seemed to be no impairment of wound healing perceivable by the bare eye. There was clear re-epithelisation of wound edges without any uncontrolled proliferation or vascularisation. This was in clear contrast to the state of the dorsal fins of the fish in YrT1/YrT2, which were observed to be damaged in the majority of fish before start of the experiment (also visible in the videos). The dorsal fins of those fish were shortened, their epithelium significantly thickened and multiple capillary blood vessels were observable.

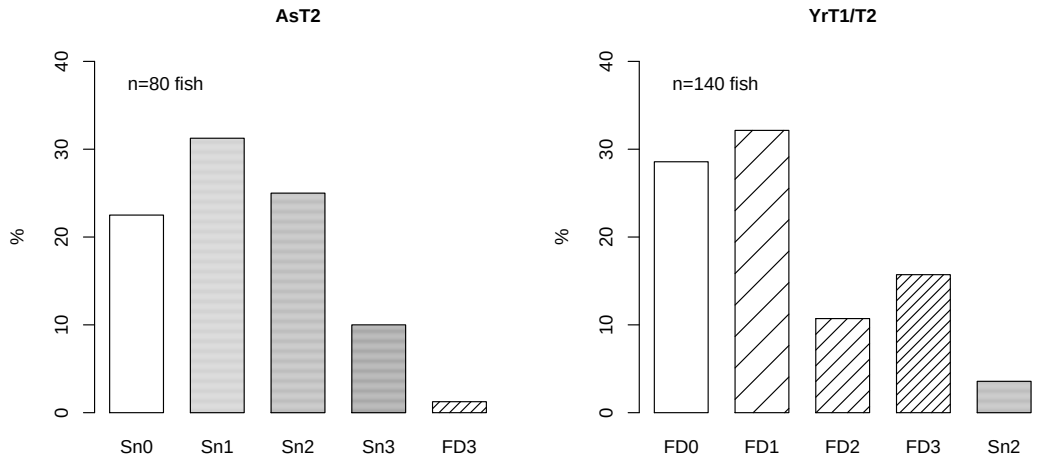

Figure 3.3: Lesions not related to infection - Main findings of gross pathology in AsT2 (left plot) and YrT1/T2 (right plot). All lesions listed were assumed *not* to be caused by infection of either *A. salmonicida* or *Y. ruckeri*. For definitions see Tab.3.1.

3.2 Video-observation

3.2.1 Selected indicators for disease

Visually perceptible traits (VPTs)

The following VPTs were chosen after exploratory observation of the recorded material: bottom contact, marginal position, fin movement, angle dorso-ventral axis, locomotion quality, collision with tank walls, stability in current, abdominal curvature and anorexia. For definitions and observation intervals (i.e. the time frame in which the respective VPT was evaluated to be absent or present) of each VPT see Tab.3.2.

Potential death predictors (PDPs):

After visual exploration of the VPT data, the following PDP were chosen: dorsal or lateral recumbency, dorsal or lateral recumbency together with severe perpendicular instability, dorsal or lateral recumbency together with severe perpendicular instability together with light perpendicular instability, passive floating, motionless on tank bottom, stiffened locomotion, tucked-up abdomen and anorexia. For detailed descriptions of each PDP see Tab.3.3. A drawing (Fig.3.4) is added to explain terms used to describe anatomical features.

3.2.2 Graphical evaluation of PDPs

Complete anorexia

Complete anorexia warranted reliable identification of nonsurvivors (Sens=100%) in both AsT1/T2 and YrT1/T2 (see Tab.3.4). The false positive rate however was quite different between the two groups. In AsT1 11% of surviving fish showed at least once complete anorexia. However, as no re-isolation of bacteria was performed during this trial, it cannot be excluded that those fish were just dying more slowly than the others. While in AsT2 complete anorexia warranted good identification of survivors (Spec=97%) in YrT1/T2 the proportion of surviving animals showing at least once complete anorexia was high. The percentage among surviving animals showing at least once complete anorexia during the course of experiment was notably higher for the two *Y. ruckeri* trials (see 3.6). Among the control animals, fish of YrT2 showed also a higher proportion

Table 3.2: Visually perceptible traits (VPTs) evaluated by video-observation

Denotation	Abbrev.	Definition	Observ. interval
Bottom contact	BC	1 Animal is positioned on the bottom of the tank, touching the tank bottom continuously for a minimum of 5 seconds. 0 Animal is not touching the tank bottom continuously for a minimum of 5 seconds. NA Presence/absence of VPT cannot be determined (e.g. animal is not identifiable, visible or in suitable position).	5 s
Marginal position	MP	1 Animal is positioned on the bottom of the tank with less than one body width distance to a lateral wall of the tank. 0 Animal is positioned on the bottom of the tank with more than one body width distance to a lateral wall of the tank. NA Animal does not show bottom contact (BC = 1), is not identifiable, visible or in suitable position.	5 s
Fin movement	Fmov	0 Animal shows perceptible movement of dorsal fin and/or pectoral fin for minimum 5 seconds. 1 Animal does not show perceptible movement of dorsal fin and/or pectoral fin for minimum 5 seconds. NA Presence/absence of VPT cannot be determined (e.g. animal is not identifiable, in suitable position or its fins are not visible).	5 s
Angle dorso -ventral axis	DVax	0° Fish being in an upright position in the water column. 45° Fish being leaned against lateral wall in a slight angle. 90° Fish being in lateral recumbency, either on tank bottom of floating just under the water surface. 180° Fish being in a belly-upwards position, either on the tank bottom or anywhere in the water column. 0-45° Instability of dorso-ventral axis, varying between 0 and 45° during 20 seconds. 0-180° Instability of dorso-ventral axis, varying between 0 and 180° during observation interval (≈ 20 s[1]). NA Presence/absence of VPT cannot be determined (e.g. animal is not identifiable, visible or in suitable position).	20 s
Locomotion	Loc	Subjective evaluation of movement pattern during locomotion. Only perceived presence of deviation from normal movement was noted[2]. 0 Either no locomotion visible or locomotion perceived as being normal. 1 Animal show slow, rocking movement with its cranio-caudal axis seeming unusually rigid (contrast to usual smooth undulating movement). 2 Fast and uncoordinated locomotion with strong movement of caudal fin (≈ 20 s[1]). NA Animal not identifiable/visible.	20 s
Collision	Col	1 Animals colliding with tank walls out of a free trajectory. 0 No collision with tank wall observed. NA Presence/absence of VPT cannot be determined (e.g. animal is not identifiable, visible or in suitable position)	≈ 20 s[1]
Stability in current	StatCur	Ability of animals to keep a position in a current. This was mainly of significance in YrT1/T2. In AsT1/T2 animals had the possibility of swimming in a weak current of the water-inlet, but could easily retreat from the current. 2 Animal is passively dragged with the current. 1 Animal is able to hold its station against the current. 0 Animal is not positioned inside a current. NA presence/absence of VPT cannot be determined (e.g. animal is not identifiable, visible or in suitable position)	20 s
Abdominal curvature	Abd	Subjective evaluation of the shape of abdominal curvature. 2 Localised bulging of abdominal wall. 1 S-shaped abdominal curvature. 0 U-shaped abdominal curvature showing no localised bulging. NA Presence/absence of VPT cannot be determined (e.g. animal is not identifiable, visible, in suitable position) *or* observer is unable to distinguish between U-shaped and S-shaped abdominal curvature.	20 s
Anorexia	Anor	1 Animal did not take up feed. 2 Animal showing low feeding activity[3]. 0 Animal was observed taking up feed.	[4]

[1] Exact timeframe was not always adhered to, because it turned out during observation that those VPTs were of brief duration, were intermittently rather than constantly shown and often happened more or less directly before or after the fixed observation interval.

[2] If no locomotion was visible it was not possible to evaluate the absence or presence of this VPT. VPT was set to Loc = 0 (although it would have been more logical to set to NA). Therefore ObNA% (one of the statistical key figures calculated) could have been calculated slightly higher.

[3] This VPT was unfortunately inconsistently rated, so for the analysis Anor = 2 was set as Anor = 0.

[4] Observation interval was variable, starting from the moment feed was introduced into the tank until all feed was consumed or no feeding activity could be observed anymore for 30 seconds.

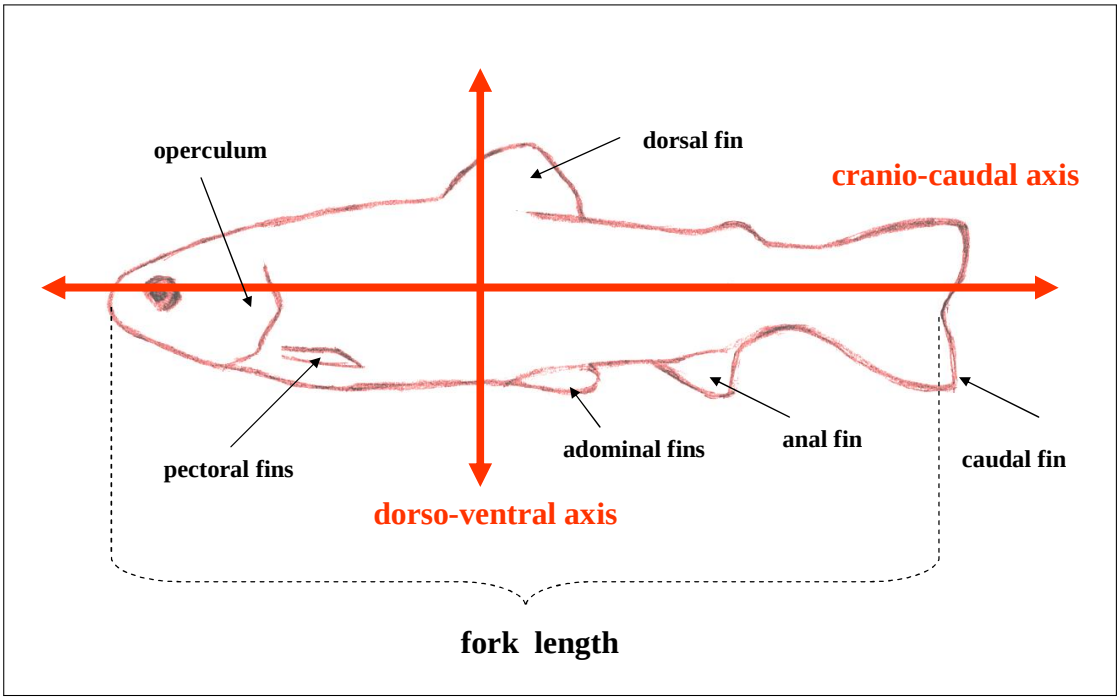

Figure 3.4: Terms used to describe anatomical features in the current text.

Table 3.3: Potential Death Predictors (PDPs) evaluated by video-observation

Denotation	**Abbr.**	**Analysed group**	**VPTs included**	**Description**
Dorsal or lateral recumbency	DLR	1) Nonsurvivors AsT1/T2 2) Nonsurvivors YrT1/T2 3) Survivors AsT1	DVax = 90° or DVax = 180°	-Animal is lying either on its side or on its back.
Dorsal or lateral recumbency or severe perpendicular instability	DLG + SevPi	1) Nonsurvivors AsT1/T2 2) Non-survivors YrT1/T2 3) Survivors AsT1	DVax = 90° or DVax = 180° or DVax = 0-180°	-Animal is lying either on its side or on its back. or -Animal shows inability to keep its dorso-ventral axis vertical. Rotations of dorso-ventral axis > 45° are observed.
Dorsal or lateral recumbency or severe perpendicular instability or light perpendicular instability	DLG + Sev/LiPi	1) Nonsurvivors AsT1/T2 2) Nonsurvivors YrT1/T2	DVax = 90° or DVax = 180° or DVax = 0-180° or DVax = 0-45° or BC = 1 and DVax = 45°	-Animal is lying either on its side or on its back. or -Animal shows inability to keep its dorso-ventral axis vertical. Rotations of dorso-ventral axis > 45° are observed. or -Animal shows inability to keep its dorso-ventral axis vertical. Rotations of dorso-ventral axis $\leq$ 45° are observed.
Passive Floating	PaFlo	1) Nonsurvivors AsT1/T2 2) Nonsurvivors YrT1/T2	StatCur = 2	Animal is observed to be passively dragged in the current and seems no longer able to maintain stationary.
Motionless on tank bottom	MLBo	1) Nonsurvivors AsT1/T2 2) Nonsurvivors YrT1/T2	BC = 1 and Fmov = 0 and (DVax $\neq$ 90° or DVax $\neq$ 180°)	Animal stays in body contact to tank bottom, without movement of pectoral and caudal fin and without showing lateral or dorsal recumbency.
Stiffened locomotion	SLoc	1) Nonsurvivors AsT1/T2 2) Nonsurvivors YrT1/T2	Loc = 1	Animal shows slow, rocking movement with its cranio-caudal axis seeming unusually rigid.
Tucked-up abdomen	TAbd	1) Nonsurvivors AsT1/T2 2) Nonsurvivors YrT1/T2	Abd = 1 or Abd = 2	-Animal shows S-shaped abdominal curvature or -Localised bulging of abdominal wall.
Complete Anorexia	cAnor	1) Nonsurvivors AsT1/T2 2) Survivors AsT1/T2 3) Nonsurvivors YrT1/T2 4) Survivors YrT1/T2	Anor = 1	Animal shows complete anorexia.

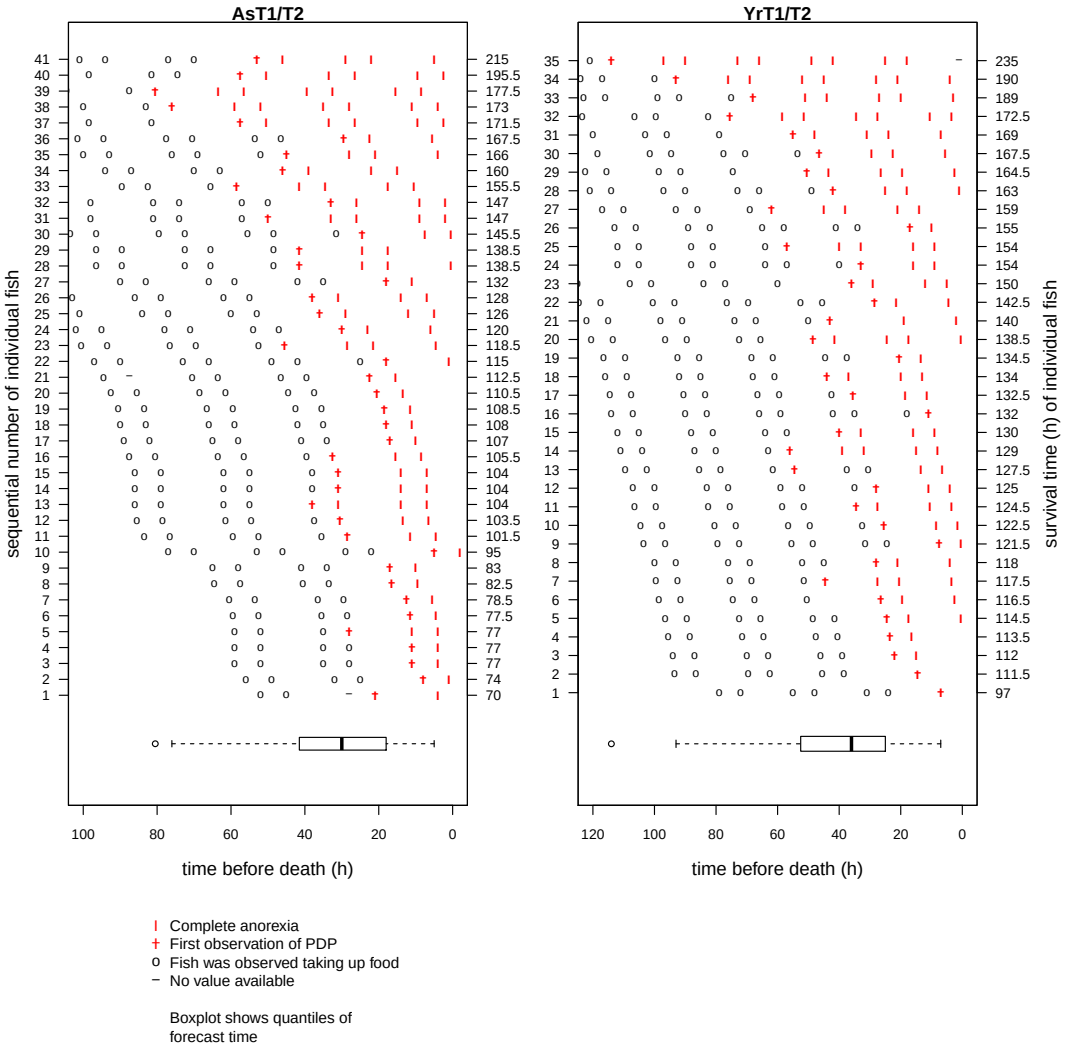

Figure 3.5: Complete Anorexia - AsT1/T1 and YrT1/T2 ; strip-charts show observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

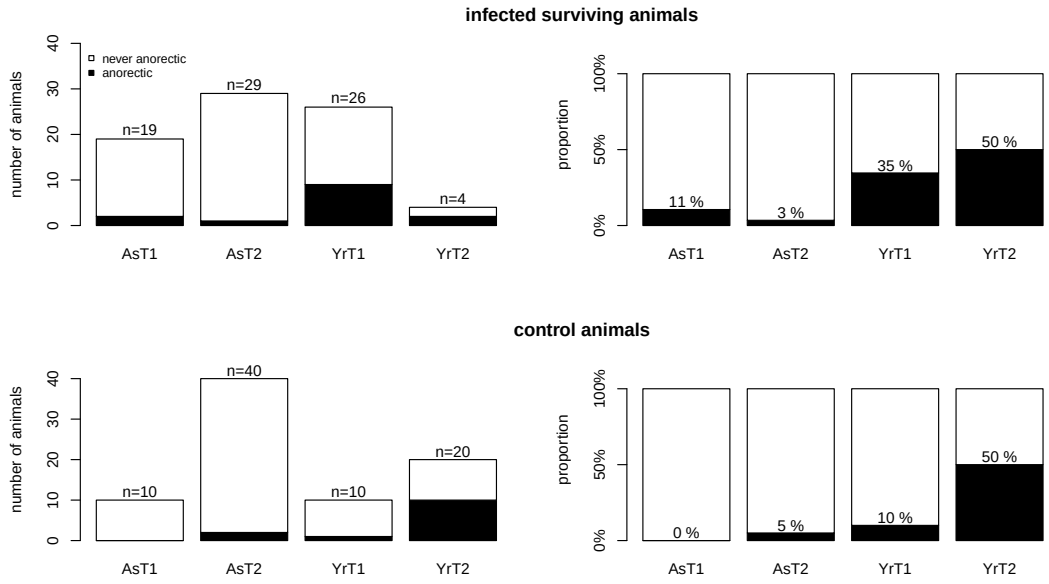

Figure 3.6: Proportions of anorectic animals among survivors and control animals - AsT1[1], AsT2 , YrT1 , YYrT2.

[1] no re-isolation performed in survivors within AsT1, therefore values have to regarded with caution.

of anorectic animals than the control animals from AsT1 and AsT2. In vaccinated animals, as well as in control animals from YrT1 the proportion of anorectic animals was slightly higher than in control animal from AsT1/T2.

Partial and complete anorexia in survivors, control animals and vaccinated animals

Survivors, control animals and vaccinated animals showed more intermittent complete anorexia, compared to nonsurvivors, (see Fig.3.7). Surviving fish, in which either *A. salmonicida* or *Y. ruckeri* was re-isolated, were sometimes observed to show continuous complete anorexia. It appeared striking to the observer that fish often showed partial or complete anorexia at the second feeding of the day, while it seemed to be less often observed at the feeding in the morning. Three fish that were euthanized or died from a different aetiology than *Y. ruckeri* or *A. salmonicida*[1] also demonstrated complete anorexia. One surviving animal showing prolonged complete anorexia (Sequential number=13 in Fig.3.7) was found to be the subordinated animal among two surviving fish that remained in the experimental tank after all other animals died and was observed to be frequently attacked by the dominant animal.

Tucked-up abdomen

Tucked-up abdomen (often associated with kyphosis) could be almost exclusively observed in fish being infected with *A. salmonicida*. In YrT1/T2 a tucked-up abdomen was determined to be present in only 3 out of 33 dying fish (in 1-2 time points per fish) and was therefore not further analysed. In AsT1/T2 this PDP warranted reliable identification of dying animals (Sens=100%). Specificity was not evaluated[2]. This PDP was present relatively early (50Q(t_{Foc})=23 h) but with high variability between animals. Variation of t_{Obs} (MAD (t_{Obs})=133) was a little higher than in

[1] For two fish, swimbladder stress syndrome was suspected. One fish was euthanized because of complete abrasion of caudal fin by nipping of dominant fish, see also Tab.A.1 in Appx.A and Fig.B.7 animal on blue background in Appx.B.

[2] However the results from graphical analysis of abdominal curvatures (see later in text) suggest that this PDP is present only in nonsurvivors.

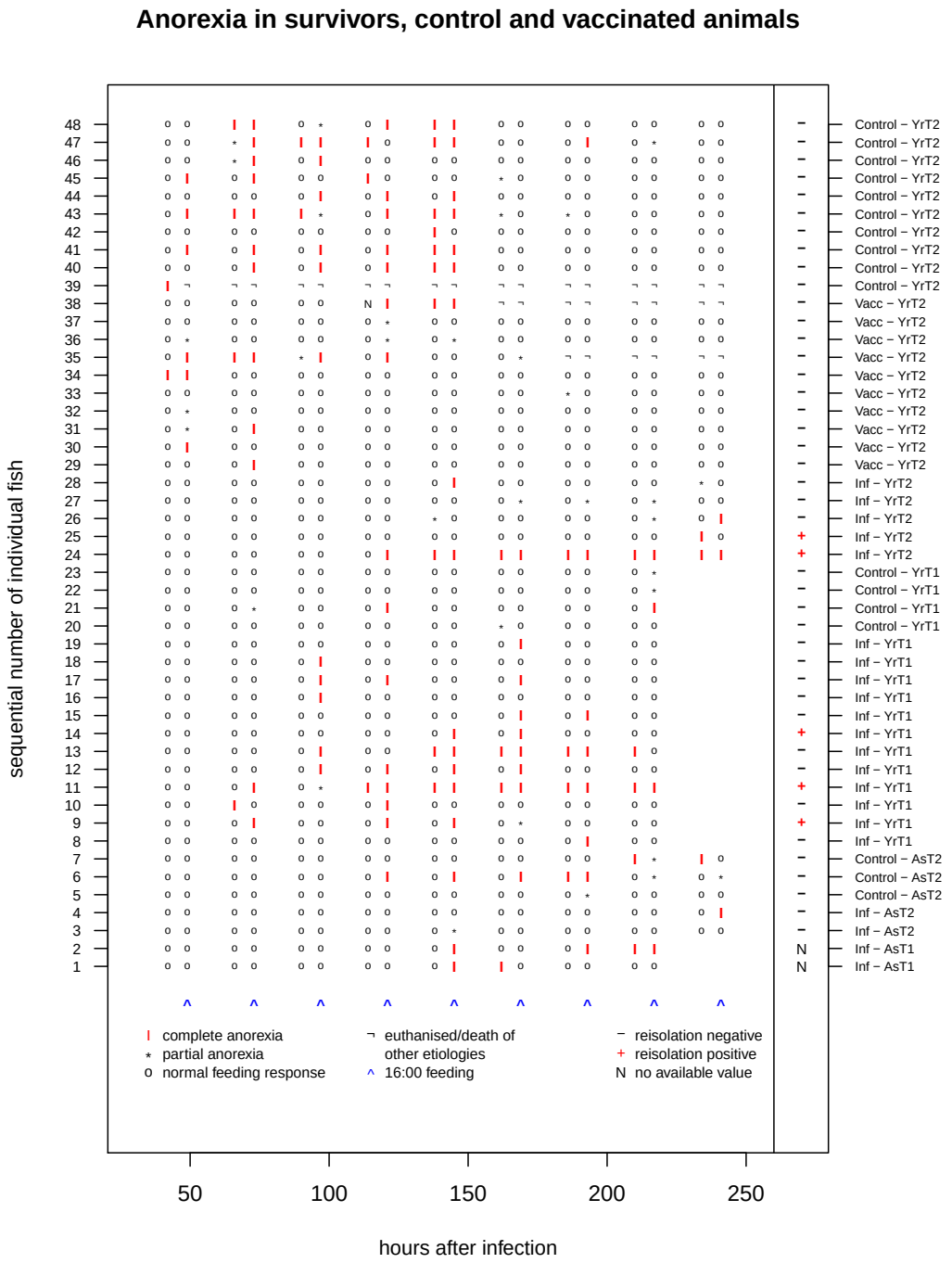

Figure 3.7: Partial and complete anorexia in survivors, control and vaccinated fish of all trials - figure shows occurrence of complete or partial anorexia relative to time of infection. Ordinate on the right side gives the group affiliation (Inf= survivor of infection; Control= uninfected control; Vacc= vaccinated animal) and respective trial. Additionally information is given about re-isolation status and eventual euthanasia.

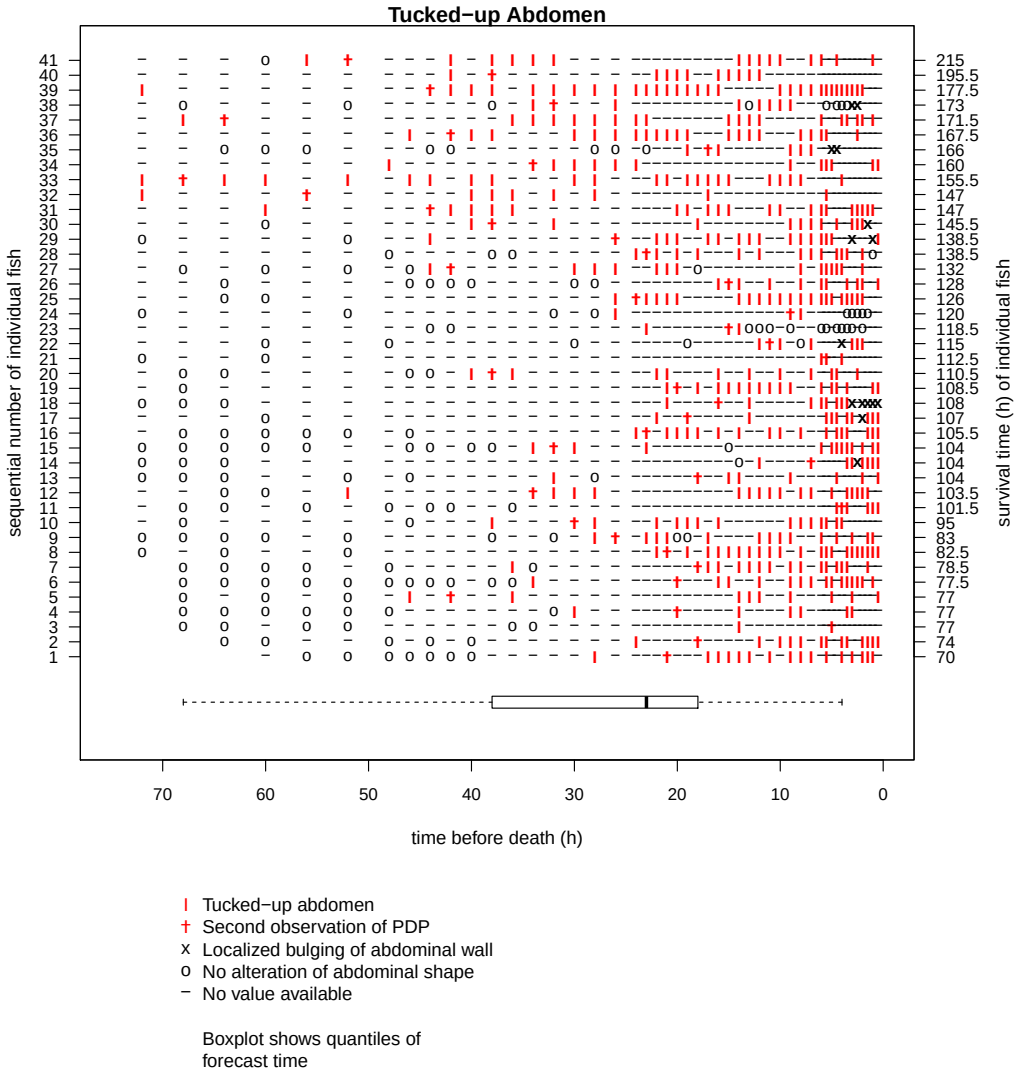

Figure 3.8: Tucked-up abdomen (TAbd) - AsT1/T2 ; Strip-chart shows observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

complete anorexia but lower than in all other VPT analysed in this group, suggesting that this PDP seems to occur more independently from survival time. It was noted that tucked-up abdomen could be observed much more frequently than other PDP assessed during the same time-schedule (see Fig.3.8). However, there seems to be some variability between the fish. This finding has to be regarded with caution, as the proportion of not evaluable observations was extremely high (50Q(ObNa%)= 62.5%). It was noticeable that some fish seemed to demonstrate an intermittent tucked-up abdomen (Fig.3.8), which could be also interpreted as a sign of low observer reliability. It seems like there could exist a positive correlation between survival time and forecast time. But since the percentage of non-available observations was considered very high, no further analysis was performed.

Settling motionless on tank bottom

In AsT1/T2, settling motionless at the bottom of the tank was frequently observed in non-surviving fish (Sens=68.2%) before showing lateral or dorsal recumbency. Unfortunately specificity was not

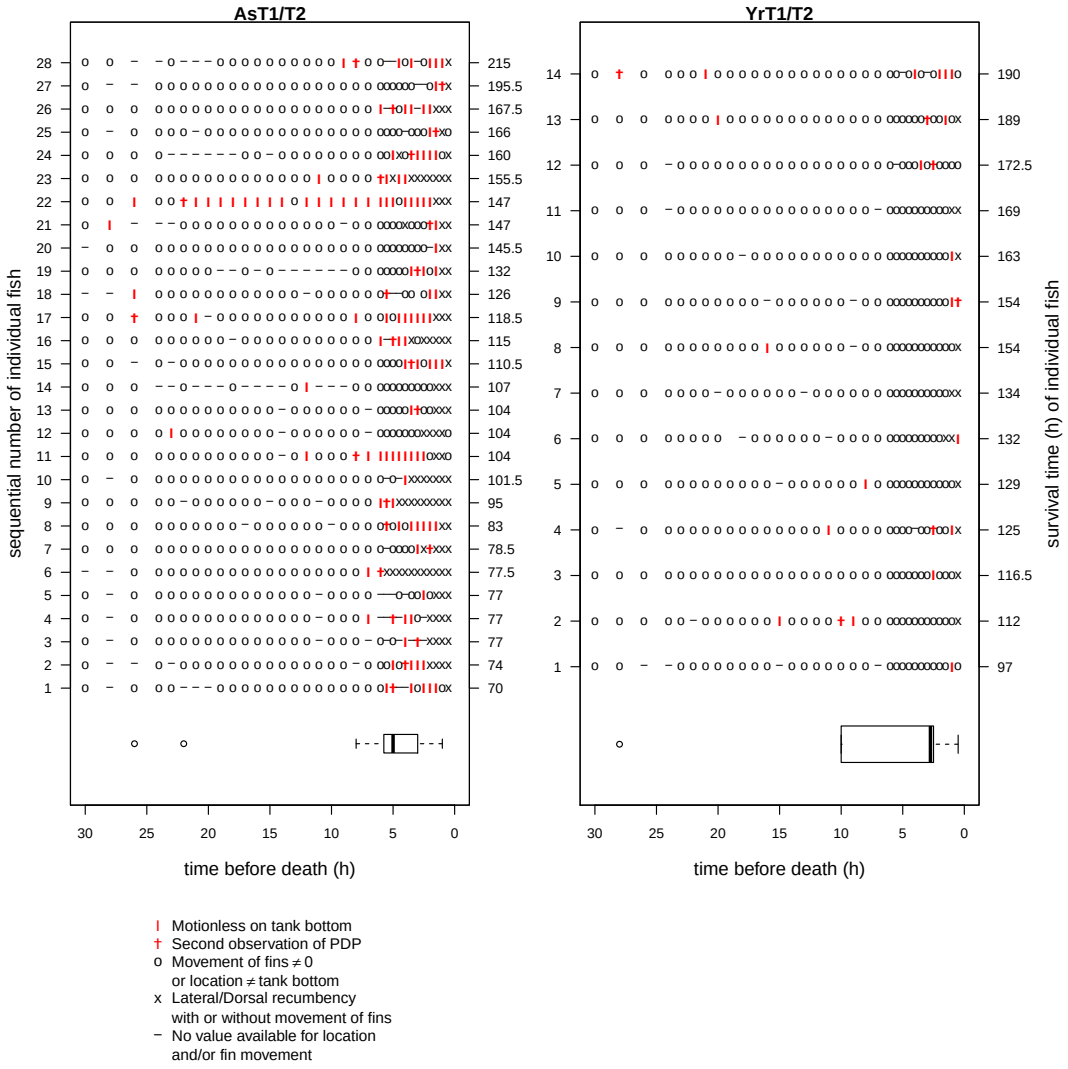

Figure 3.9: Motionless on tank bottom (MLBo) - AsT1/T2 and YrT1/T2 ; Strip-charts show observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

assessed for this PDP. Forecast time seemed overall relatively short (50Q(t_{Foc})=5 h) and did not vary heavily between fish (MAD(t_{Foc})=4) in AsT1/T2. Fig.3.9 shows that many fish demonstrated this PDP intermittently. Therefore it was chosen to use the second observation for calculating t_{Foc}. In YrT1/T2 less nonsurvivors showed this trait (Sens=42.4%). Also, it seemed to be observed less per individual animal (see Fig.3.9) and forecast time was even shorter (50Q(t_{Foc})=2.75 h) than in AsT1/T2. Proportion of non-available observations was highest among all other PDP analysed in YrT1/T2 (50Q(NaObs%)=6.25%).

Stiffened locomotion

In AsT1/T2 SLoc could be observed quite frequently and with high continuity until death in some fish, while in others this PDP was observed only once or twice, in varying proximity to death (more intermittent than continuous). In YrT1/T2 SLoc was observed only once in most of the fish in close proximity to death (see Fig.3.10). 50Q(t_{Foc}) was 4.25 hours for AsT1/T2, while in YrT1/T2 it was only 1 hour. Sensitivity in AsT1/T2 was 82.93%, in YrT1/T1 it was only 51.51%.

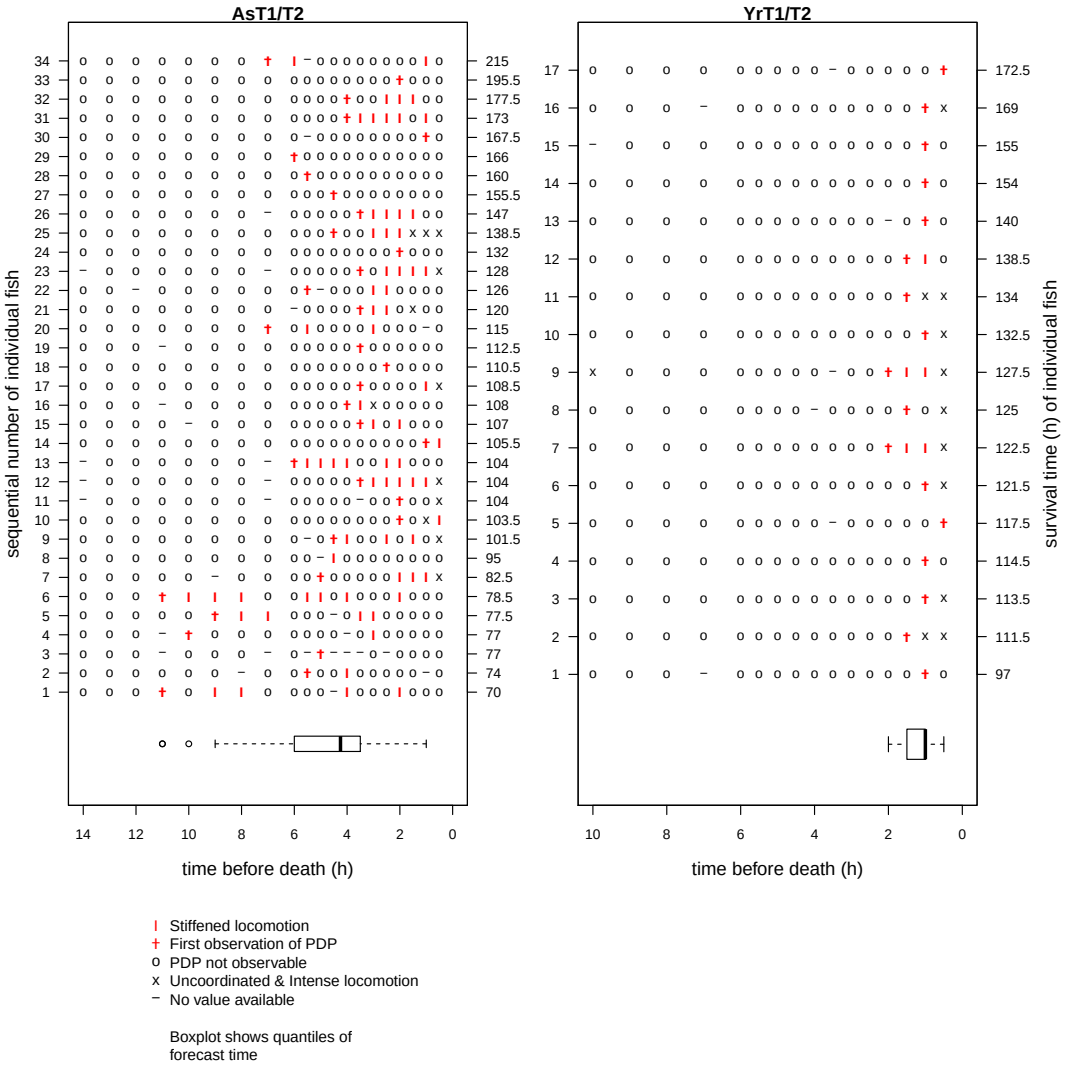

Figure 3.10: Stiffened locomotion (SLoc) - AsT1/T2 and YrT1/T2 ; Strip-charts show observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

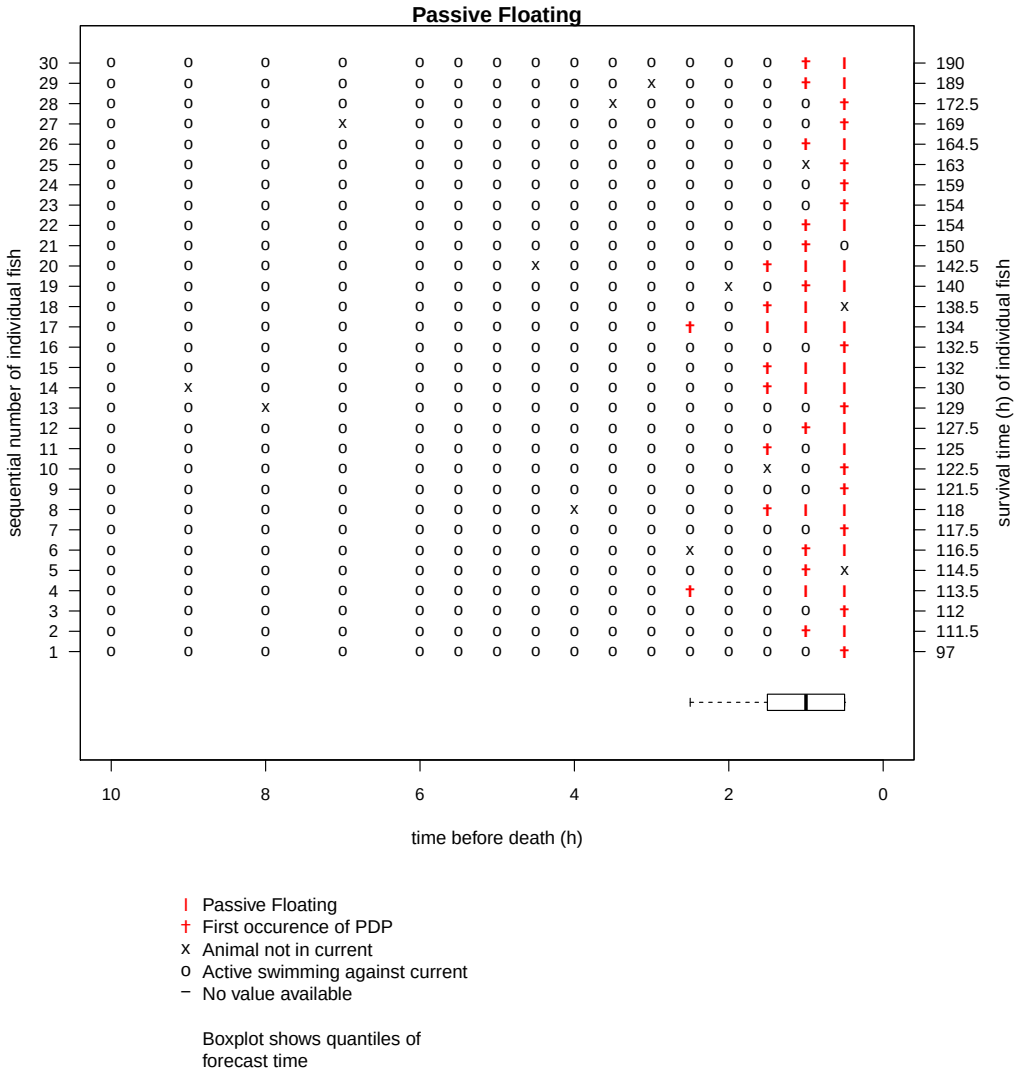

Figure 3.11: Passive Floating (PaFLo) - YrT1/T2 ; Strip-chart shows observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

Dorsal/Lateral recumbency, severe/light perpendicular instability, passive floating

Because of their similar characteristics those PDPs are reported together. In AsT1/T2 DLR, DLR+sevPi and DLR+sev/LiPi could be observed with high continuity until death (see Fig.3.12), meaning that once an animal demonstrated those PDP, the probability was high it would show this PDP again at the following observation until death. The same was true for YrT1/T2, but as t_{Foc} was much shorter, the continuity might not be of practical relevance in this case (see Fig.3.13). Passive Floating was only assessed in YrT1/T2 and seemed to be extensively interconnected with DLR and DLR+sevPi (see Fig.3.11). All of those PDPs demonstrate a short forecast time (for detailed values see Tab.3.4), being little longer in AsT1/T2 compared to YrT1/T2. Sensitivity was overall high, except for DLR in YrT1/T2 (Sens= 54%).

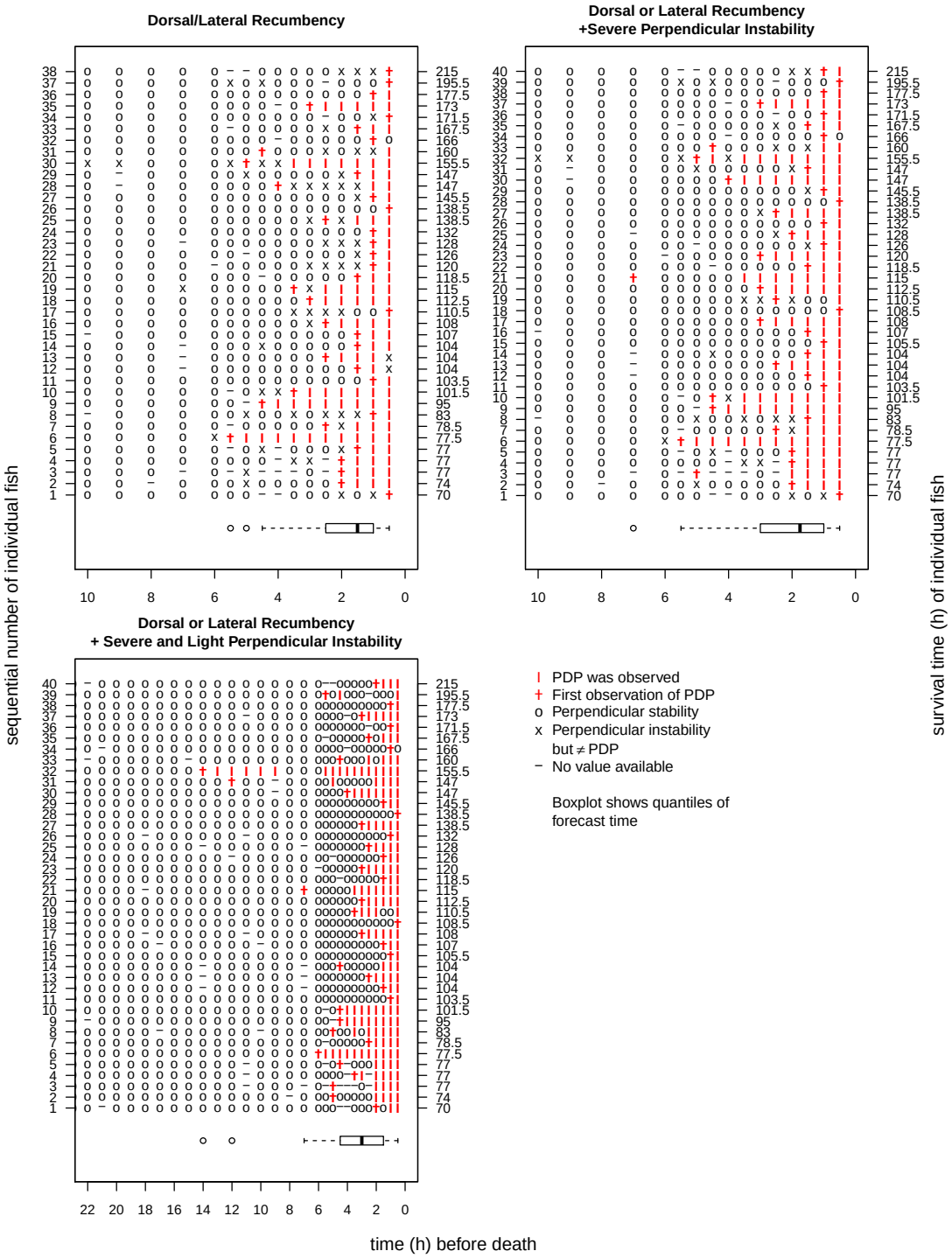

Figure 3.12: AsT1/T2 - DLR , DLR+sevPi, DLR+sev/LiPi; Strip-charts show observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

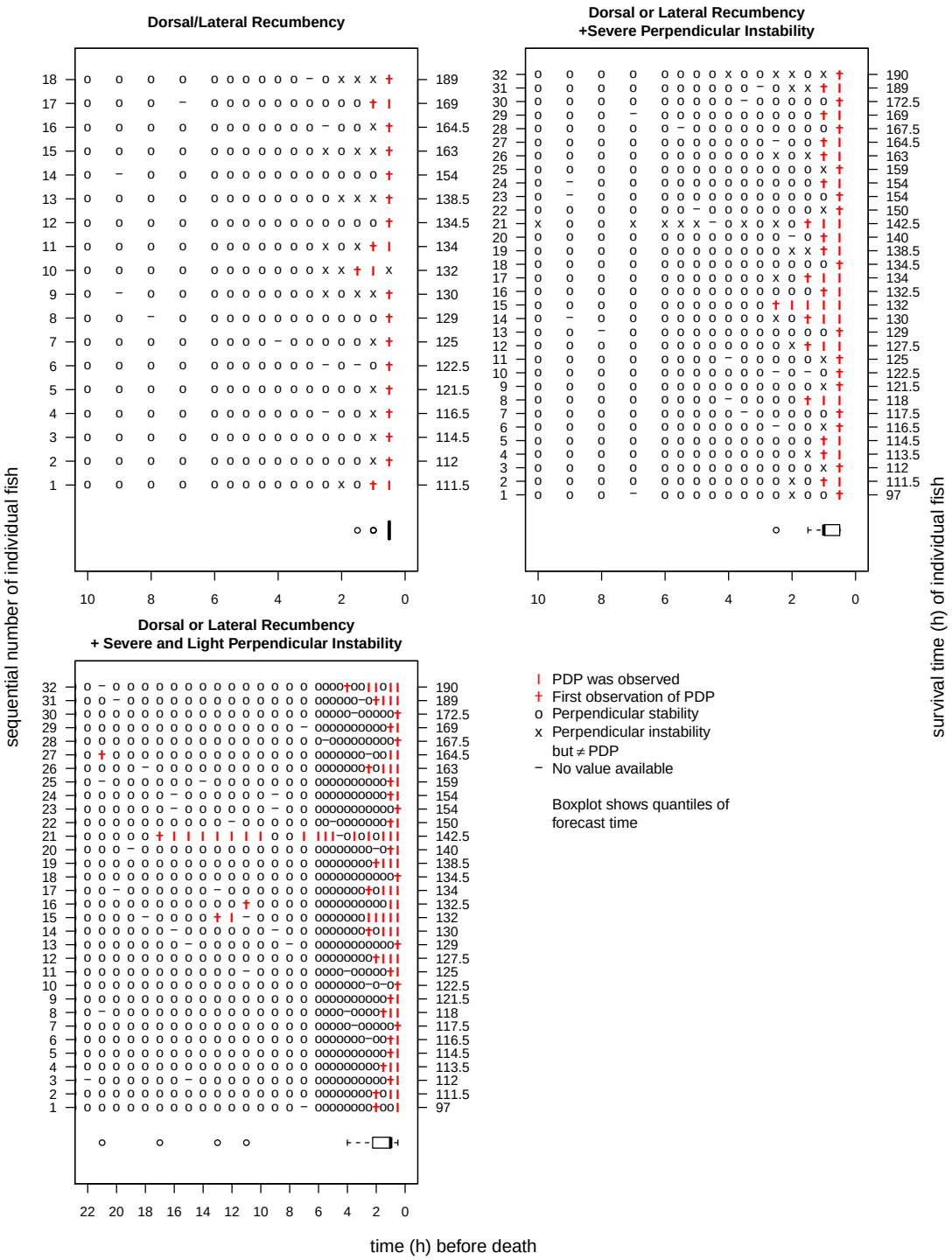

Figure 3.13: YrT1/T2 - DLR , DLR+sevPi, DLR+sev/LiPi; Strip-charts show observed presence or absence of respective PDP relative to time of death in individual fish. The left ordinate of each plot gives the sequential number of the individual fish that showed the PDP of interest. The abscissa gives the countdown time to death. The right ordinate gives the time between infection and death of each individual (survival time). Animals are sorted by survival time (increasing from bottom to top).

3.2.3 Numerical analysis of PDPs

Tab.3.4 gives the statistical key figures calculated for each PDP, described in Chap.2, Tab.2.4.

Comparison between PDPs

Among all evaluated PDPs it seems that cAnor was the earliest predictor of death in both AsT1/T2 and YrT1/T2, followed by TAbd in AsT1/T2. However, cAnor as well as TAbd had the highest variance of t_{Foc}. The four latest predictors of death were DLR, DLR+sevPi and DLR+sev/LiPi together with PaFlo in YrT1/T2. In those PDPs the variance in t_{Foc} was small, meaning that time of death could be predicted with higher precision than in the early predictors. MLBo was found to indicate death only slightly earlier than DLR, DLR+sevPi and DLR+sev/LiPi and PaFlo. SLoc predicted death slightly earlier than DLR, DLR+sevPi and DLR+sev/LiPi in AsT1/T2 but not in YrT1/T2. TAbd included by far the highest proportions of non-available observations, while DLR, DLR+sevPi and DLR+sev/LiPi and PaFlo seemed to have warranted good recognisability under this experimental setup. For a graphical overview see Fig.3.14 and Fig.3.15.

Table 3.4: Statistical key figures calculated for each PDP

AsT1/T2	Potential Death Predictor (PDP)						
Key statistic	DLR	DLR + sevPi	DLR + sev/LiPi	SLoc	MLBo	TAbd	cAnor
25Q(t_{Foc})	1	1	1.5	3.5	3	18	18
50Q(t_{Foc})	1.5	1.75	3	4.25	5	23	30
75Q(t_{Foc})	2.5	3	4.5	5.875	5.75	38	41.5
MAD(t_{Foc})	1	1	1	3	4	31	42
25Q(t_{Obs})	98.875	100.375	98.875	97.25	77	72	73
50Q(t_{Obs})	114.25	108.75	108.75	106.5	107	92	90
75Q(t_{Obs})	145.25	144.75	141.875	141.125	147.25	111	97
MAD(t_{Obs})	166	158	158	155	156	133	130
n_{dead}	41	41	41	41	41	41	41
nPDPdead	38	40	40	34	28	41	41
Sensitivity	92.68	97.56	97.56	82.93	68.29	100	100
nsurv	31[1]	31[1]	NA	NA	NA	NA	29[2]
nnoPDPsurv	31[1]	31[1]	NA	NA	NA	NA	28[2]
Specitivity	100[1]	100[1]	NA	NA	NA	NA	97[2]
50Q(ObNA%)	0	0	0	4.17	8.33	62.5	NA
MAD(ObNA%)	3	3	3	3	9	90	NA

YrT1/T2	Potential Death Predictor (PDP)						
Key statistic	DLR	DLR + sevPi	DLR + sev/LiPi	SLoc	PaFlo	MLBo	cAnor
25Q(t_{Foc})	0.5	0.5	1.0	1.0	0.5	2.5	25.0
50Q(t_{Foc})	0.50	1.00	1.00	1.00	1.00	2.75	36.00
75Q(t_{Foc})	0.500	1.000	2.125	1.500	1.375	8.250	52.500
MAD(t_{Foc})	2	1	1	1	1	2	50
25Q(t_{Obs})	121.25	120.00	118.50	117.00	118.00	130.25	90.00
50Q(t_{Obs})	130.00	132.00	128.00	125.50	131.00	157.75	97.00
75Q(t_{Obs})	149.625	154.750	153.125	139.000	153.375	168.000	114.000
MAD(t_{Obs})	190	193	187	183	191	231	141
n_{dead}	33	33	33	33	33	33	35
nPDPdead	18	32	32	17	30	14	35
Sensitivity	54.55	96.97	96.97	51.52	90.91	42.42	100.00
nsurv	NA	NA	NA	NA	NA	NA	30
nnoPDPsurv	NA	NA	NA	NA	NA	NA	19
Specitivity	NA	NA	NA	NA	NA	NA	63
50Q(ObNA%)	0	0	0	2.08[3]	0	6.25	NA
MAD(ObNA%)	3	3	3	3[3]	3	6	NA

[1] Assessment only in AsT1.

[2] Values calculated only for AsT2, because in AsT1 no re-isolation was performed (see Material and Methods).

[3] If no locomotion was visible it was not possible to evaluate the absence or presence of this PDP. PDP was set to SLoc = 0 (although it would have been more logical to set to `NA`). Therefore ObNA% could have been calculated slightly higher.

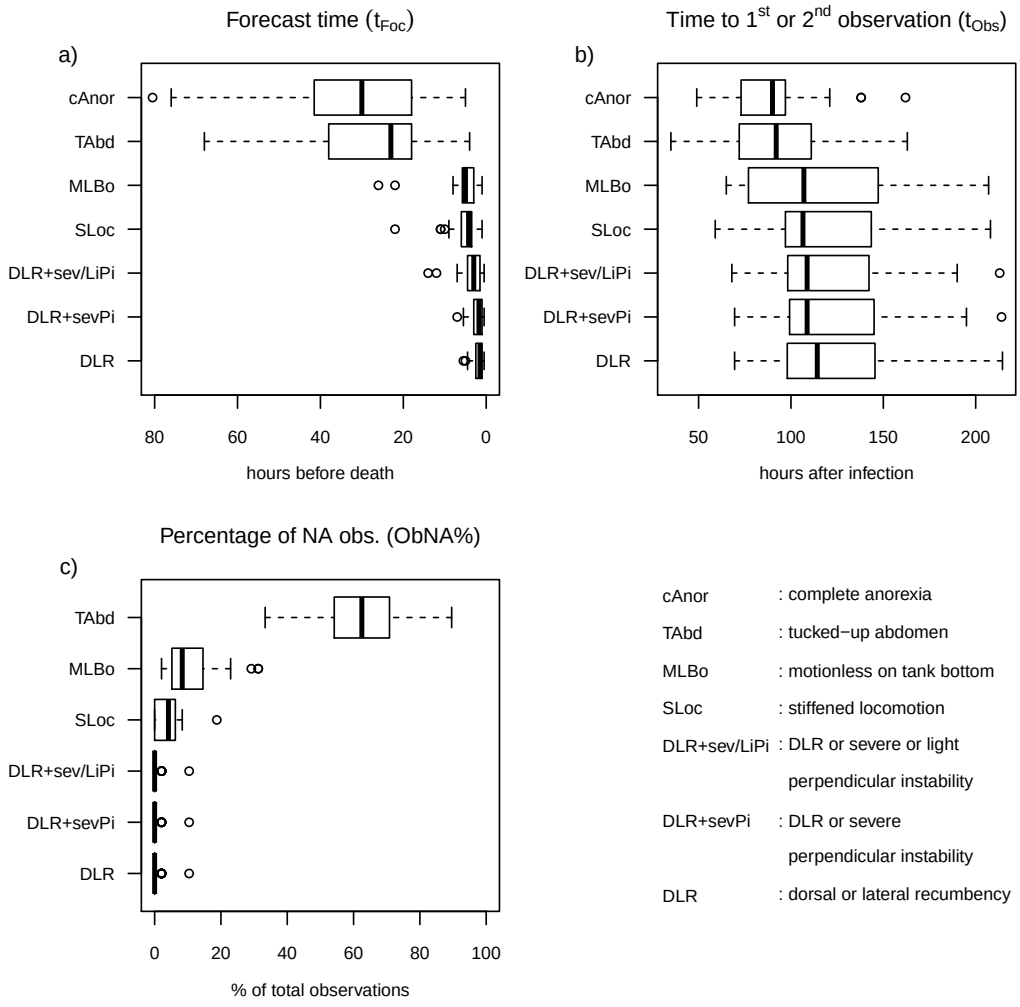

Figure 3.14: Comparison of the distribution of statistical key figures between PDPs - AsT1/T2 ; a) depicts distribution of individual forecast time for each PDP; b) shows distribution of the time between bacterial infection and first or second observation of PDP; c) shows the proportion of non-available observations from total number of observations (for more detailed description see Tab.2.4.

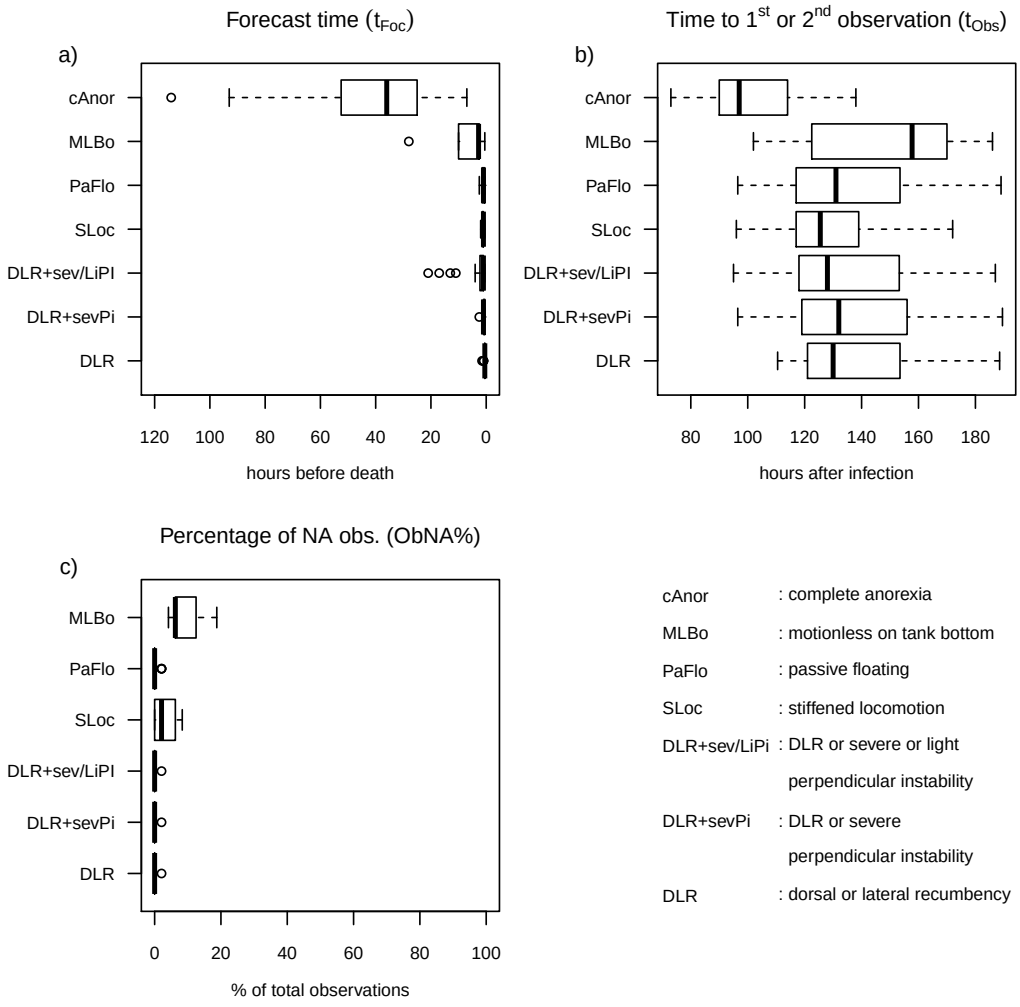

Figure 3.15: Comparison of the distribution of statistical key figures between PDPs - YrT1/T2 ;a) depicts distribution of individual forecast time for each PDP; b) shows distribution of the time between bacterial infection and first or second observation of PDP; c) shows the proportion of non-available observations from total number of observations (for more detailed description see Tab.2.4.

3.2.4 Intra-observer reliability

Intra-observer correlation was highest for Anorexia and DLR, followed by PaFlo. Results from LiPi were less good reproducible by the observer, as were SLoc and MLBo. The highest rate of disagreement between the first and the second, blinded observation was found in TAbd (Tucked-up abdomen). Complete results and details are given in Tab.3.5.

Table 3.5: Results and Details on Intra-Observer Reliability. The last two columns give the confidence intervals (CI) of Cohen's Kappa.

PDP	**Group**	**VPT Positive**	**VPT Negative**	**Nr. of videos**	**False pos.**	**False neg.**	**Cohen's κ**	**CI low**	**CI upr**
cAnor	AsT1/T2 & YrT1/T2	Anor = 1	Anor = 0	48	0	0	1	1	1
DLR	AsT1/T2	DVax=90°or DVax=180°	DVax = 0	44	0	0	1	1	1
DLR	YrT1/T2	DVax=90°or DVax=180°	DVax = 0	43	0	0	1	1	1
PaFlo	YrT1/T2	StatCur=2	StatCur=1	47	1	0	0.957	0.875	1
LiPi	YrT1/T2	DVax = 0-90°	DVax = 0	45	0	2	0.911	0.791	1
LiPi	AsT1/T2	DVax = 0-90°	DVax = 0	43	2	1	0.86	0.708	1
SLoc	AsT1/T2	Loc =1	Loc=0	48	2	2	0.833	0.677	0.99
MLBo	AsT1/T2	BC=1and FMov=0	BC=1 FMov=1	46	3	1	0.826	0.664	0.988
TAbd	AsT1/T2	Abd =1	Abd = 0	47	3	2	0.787	0.611	0.963

3.2.5 Additional numerical and graphical analysis regarding anorexia

Correlation between damage and complete anorexia

Overall, surviving fish from AsT2 showed significantly less complete anorexia than survivors from YrT1/T2. The same could be observed in the control group, where proportion of anorectic fish was significantly higher in YrT1/T2 fish (see Fig.3.16). In AsT2 54% of the surviving animals exhibited medium to severe damage (cranial/caudal lesions degree 2-3) without ever showing anorexia. The only fish showing anorexia demonstrated fin or snout lesions. No statistically significant difference in the proportion of damaged animals could be detected between the anorectic and non-anorectic group. In control animals 24% of non-anorectic animals had damaged fins or snouts and the two single animals showing anorexia had lesions as well. No statistically significant difference (p-value = 0.07; 95% Confidence interval = 0.794 – Inf.) in the proportion of damaged animals could be detected between anorectic and non-anorectic group (see Fig.3.17). However, it has to be noted there were only two anorectic animals in total, so it might be possible that the number of animals was too small to be able to prove a difference. In YrT1/T2 the proportion of damaged fish was not statistically different between anorectic survivor fish and non-anorectic survivor fish, which was according to the expectations. In vaccinated and control animals, the proportion of fish with damage seems to differ between anorectic and non-anorectic fish (see Fig.3.18). In the control animals a significant difference in the proportion of damaged animals could be detected between the anorectic and the non anorectic group. In vaccinated animals a statistical difference between proportion of damaged animals in anorectic fish and non-anorectic fish could be detected (see Fig.3.18).

Correlation between tsurv and t_{Foc}Anor/t_{surv}

Results of least-squares linear regression suggests a positive correlation between survival time (t_{surv}) and relative forecast time (t_{Foc} t_{surv}). For detailed results see Fig.3.19. This indicates that "slow-dying" animals might be proportionally longer anorectic than "fast-dying" animals.

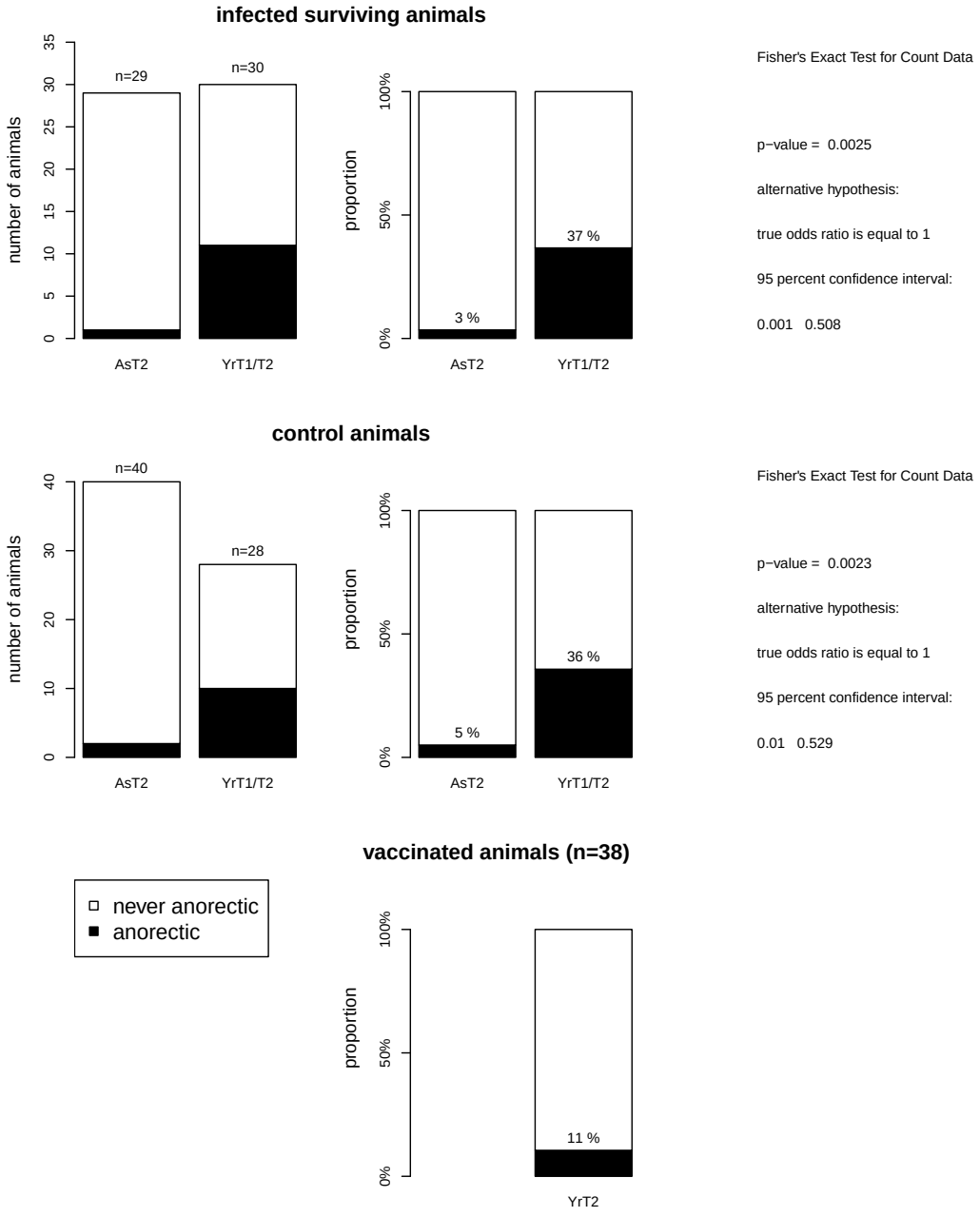

Figure 3.16: Comparison of proportion of anorectic animals between i) survivors from AsT2 and YrT1/T2 (upper barplots) plus results and details from Fisher's exact test on the right; ii) control animals AsT2 and YrT1/T2 (middle barplots) plus results and details from Fisher's exact test on the right; lower barplots show proportion of anorectic animals among vaccinated group in YrT2.

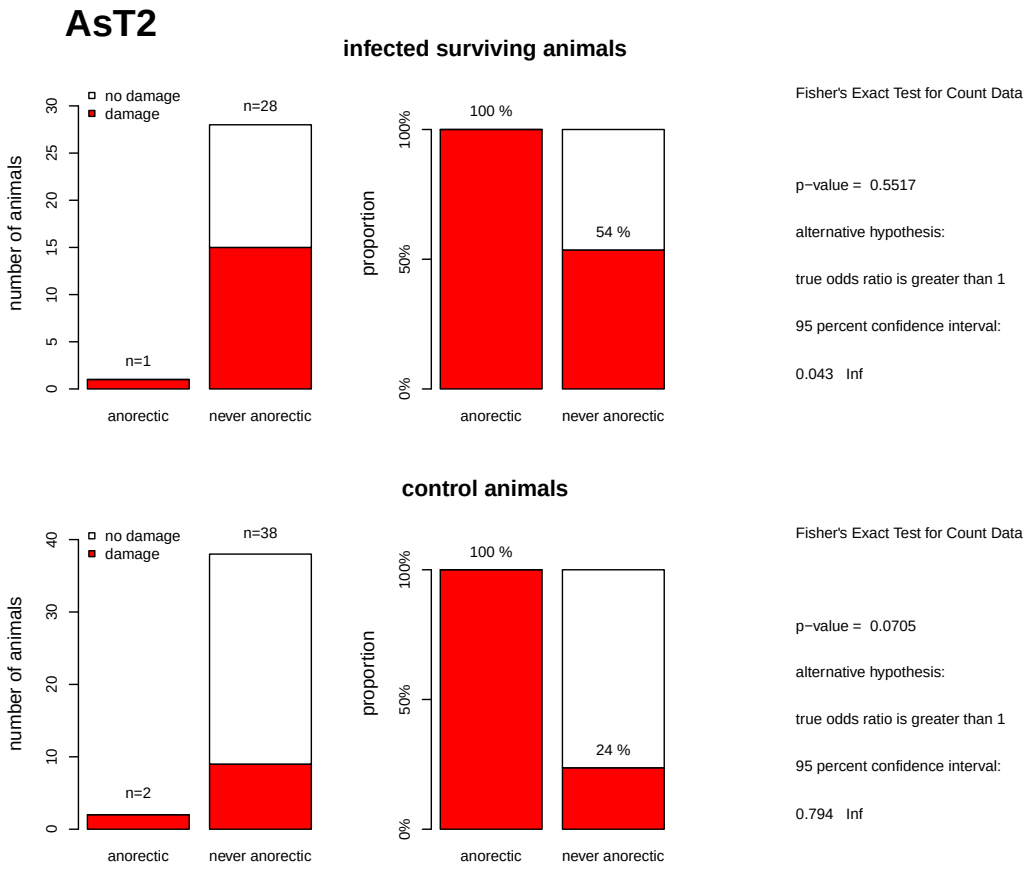

Figure 3.17: Comparison of proportion of animals with damage (degree 2-3) between anorectic and non-anorectic animals in AsT2 i) survivors (upper barplots); ii) control animals (lower barplots); results and details from Fisher's exact test on the right.

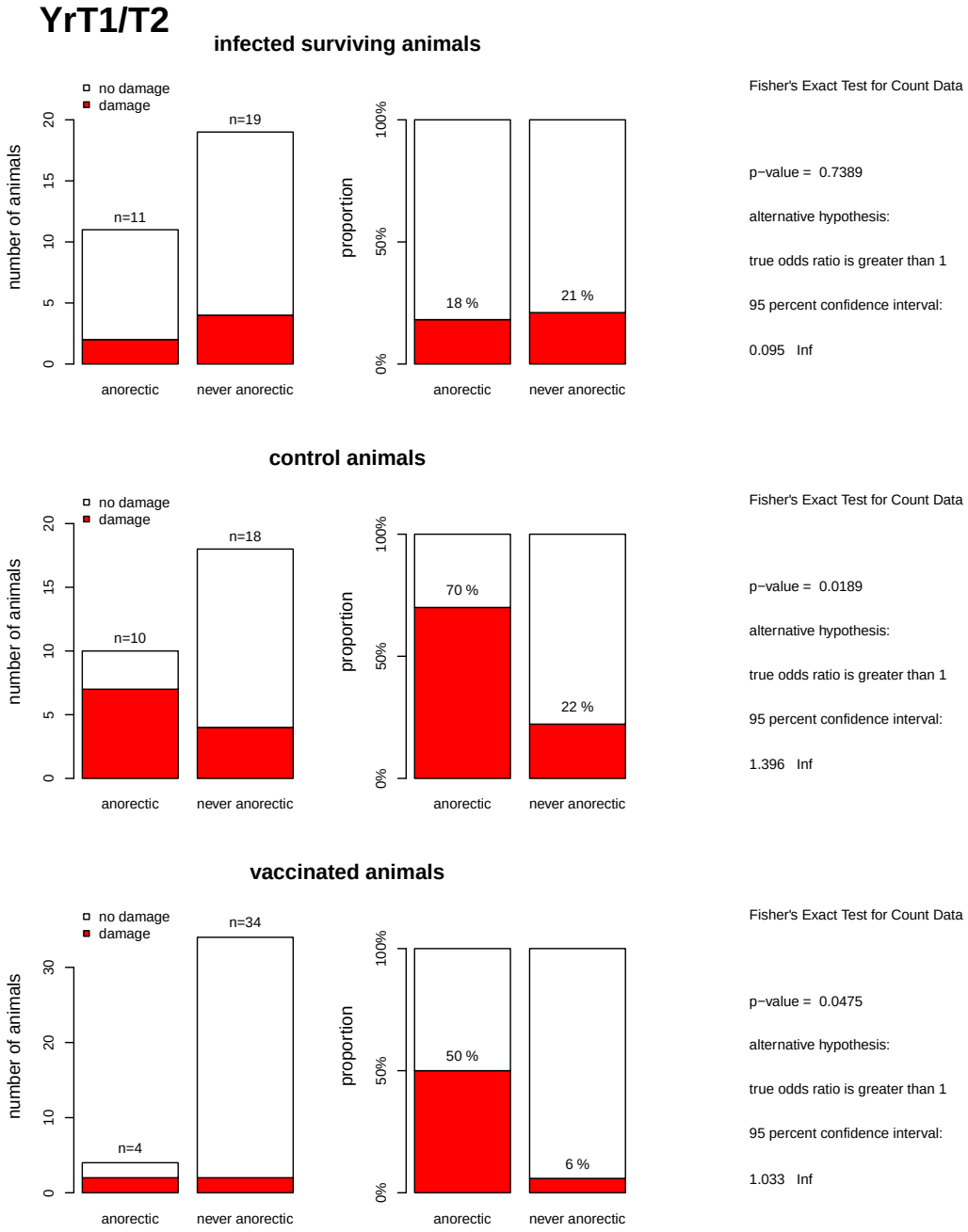

Figure 3.18: Comparison of proportion of animals with damage (degree 2-3) between anorectic and non-anorectic animals in YrT1/T2 i) survivors (upper barplots); ii) control animals (middle barplots); iii) vaccinated animals (lower barplots); results and details from Fisher's exact test on the right.

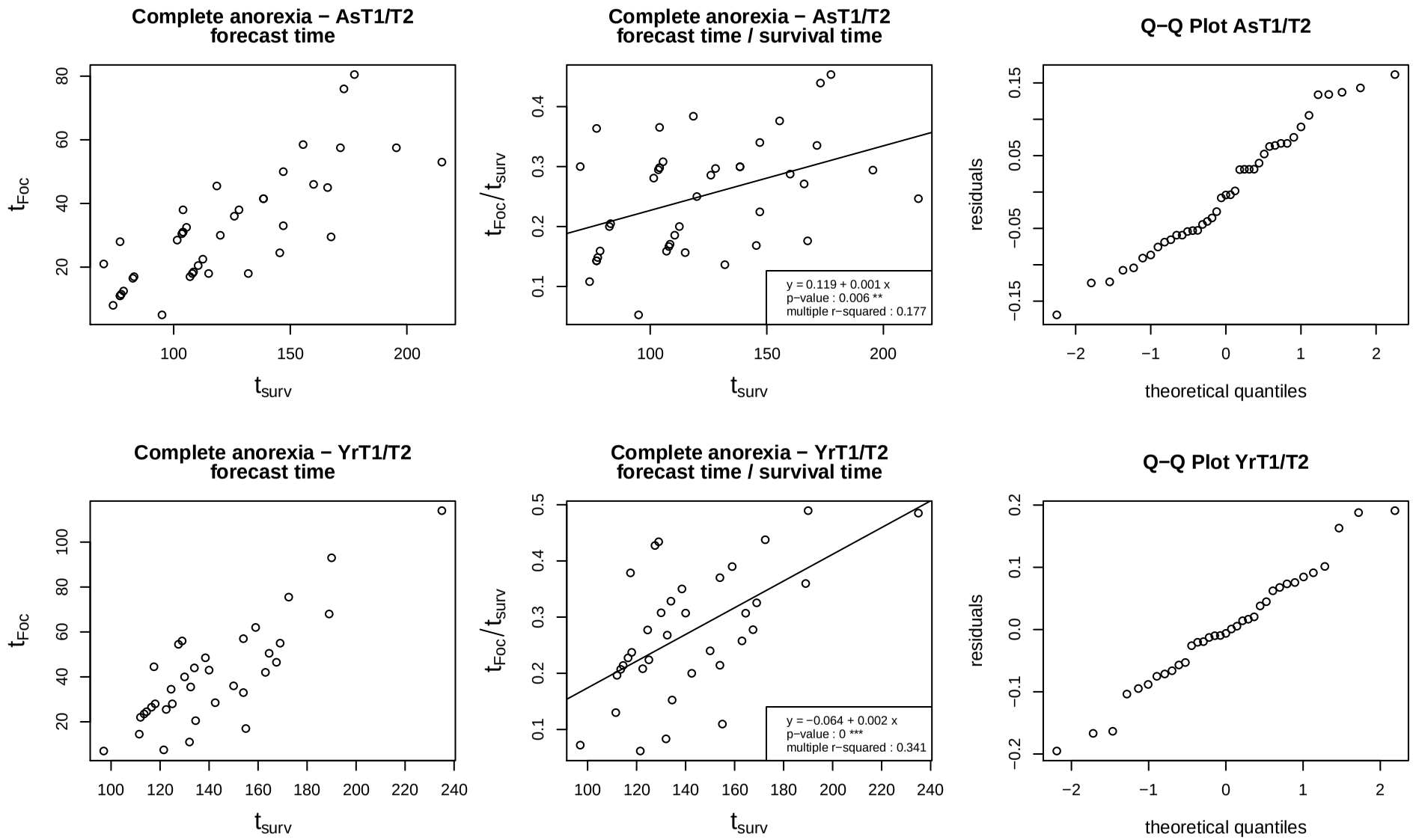

Figure 3.19: Correlation between survival time and forecast time of complete Anorexia; results of least-squares linear regression are given inside text boxes.

3.2.6 Additional graphical analysis regarding tucked-up abdomen

Abdominal (ventral) curvature

Fig.3.20 a) shows a marked difference in shape between the abdominal curvatures of non-survivors assessed early in the experiment and the abdominal curvatures of nonsurvivors assessed shortly before death, the "early curvature" corresponding to a shallow U-shape while the "late curvature" resembles an S-shape. Fig.3.20 b) again shows a marked difference in shape between the abdominal curvatures of survivors assessed early in the experiment and the abdominal curvatures of survivors later after bacterial challenge. The early curvatures of survivors resemble the early curvatures of non-survivors (Fig.3.20 c)). The late curvature of survivors takes a more pronounced U-shape. Fig.3.20 d) shows the difference in shape between the late curvatures of survivors and non-survivors. Non-survivors express a more S-shaped curvature compared to survivor's U-shaped curvature. This finding supports the impression made by direct observation that non-surviving fish, in contrast to surviving animals show a tucked-up (or S-shaped) abdomen during the course of the trial. Examples of snapshots of nonsurvivors are given in Appx.B Fig.B.8.

Dorsal curvature

Fig.3.21 a) shows a difference in shape between the dorsal curvatures of nonsurvivors assessed early in the experiment and the dorsal curvatures of nonsurvivors assessed shortly before death. Compared to the "early curvature" the "late curvature" is more convex. There was no marked difference between early and late curvatures of survivors (Fig.3.21 b)). Furthermore, no difference could be seen between early curvatures of survivors and early curvatures of non-survivors (Fig.3.21c)). Late curvatures of survivors and late curvatures of non-survivors however differed in the degree of convexity. This finding supports the impression made by direct observation that non-surviving fish, in contrast to surviving animals show kyphosis (also called "arched" back) during the course of the trial (Fig.3.21d)). Example of snapshots of nonsurvivors are given in Appx.B Fig.B.8.

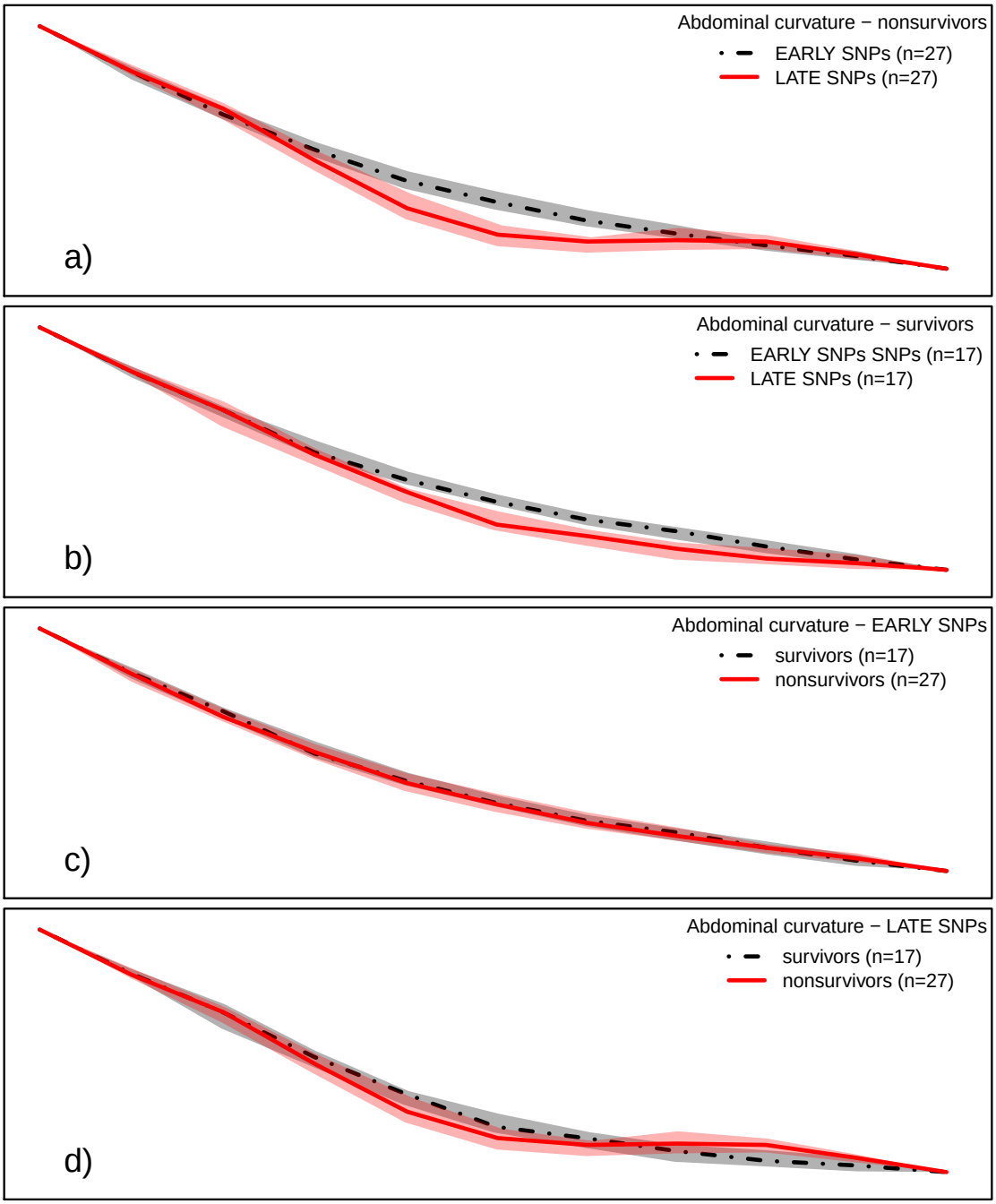

Figure 3.20: Graphical analysis of abdominal (ventral) curvature; SNPs = Snapshots ; "EARLY" SNPs refers to coordinates that were obtained from snapshots taken at begin of infection trial (see Fig.2.3; "LATE" SNPs refers to coordinates that were obtained from snapshots taken either before death (nonsurvivors) or at time of death of tank-mates (survivors) (see Fig.2.3 and explanation in chapter 2).

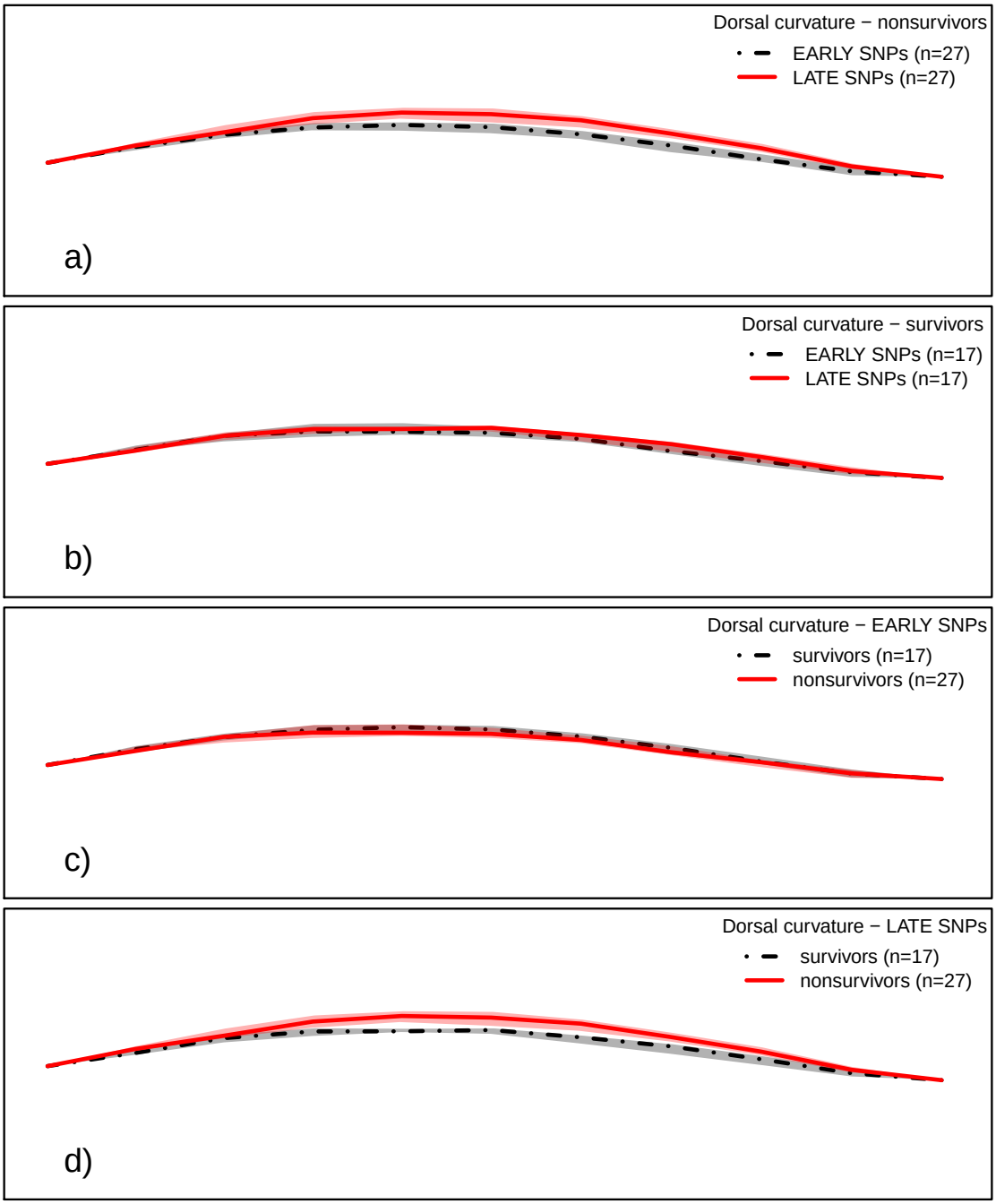

Figure 3.21: Graphical analysis of abdominal dorsal curvature (back line); SNPs = Snapshots ; "EARLY" SNPs refers to coordinates that were obtained from snapshots taken at begin of infection trial (see Fig.2.3; "LATE" SNPs refers to coordinates that were obtained from snapshots taken either before death (nonsurvivors) or at time of death of tank-mates (survivors) (see Fig.2.3 and explanation in chapter 2).

3.2.7 Miscellaneous findings of video-observation

Emesis

Emesis of fine particulate material was observed multiple times in fish from all trials as incidental finding. During video-observation the presence of fine particulate material floating in the water was occasionally noted. After rewinding the video to find the source of the particles, it turned out they originated from vomiting fish. Emesis was only observed in infected fish, both in survivors as well as non-survivors. It was never observed in control animals or vaccinated animals, although it has to be emphasised that those groups were by far not as intensively monitored as the fish that received bacterial challenge. It was distinguished from regurgitation as the particle size was much smaller than the pellet size of the feed, which suggests that the food already started to be digested before being thrown up again. Although vomiting seemed to happen quite regularly, it was unlikely to be observed directly as its duration was short (only a couple of seconds). It was therefore not considered as a PDP and was not systematically documented.

Dark colouration of skin

It was originally not considered possible to detect darkening of the rainbow trout skin colouration. It was assumed that the change in colour might be too subtle to be reliably seen, as due to limited spatial conditions, the infrared spots had to be installed in small distance to the water surface, which led to a relatively uneven illumination. However in multiple cases (in AsT1/T2 as well as YrT1/T2) darkening of non-surviving fish, shortly before death, was noticed by the observer. It has to be mentioned that under infrared light, dark colour is frequently perceived as light colour. In the case of the darkening fish, the animals were perceived to be of much lighter colour than their tank-mates.

Increased frequency of opercular movement

In both AsT1/T2 as well as in YrT1/T2 it appeared to the observer that non-surviving fish at some point before their death exhibited increased frequency of opercular movement (ventilation rate). Opercular movement also seemed more pronounced, meaning the opercula were being spread outwards to a higher degree compared to surviving tank-mates. Subjectively this phenomenon was observed to be more noticeable in YrT1/T2 than in AsT1/T2. Unfortunately, out of timely restraints, this subjective observation could not be numerically validated, for example by counting the frequency per minute.

Protruding anus

Protrusion of anus was observed regularly in fish in AsT1/T2 showing abdominal distension but was not systematically assessed.

Social aggression and formation of a social hierarchy

Intra-specific aggression in form of chasing and nipping could be observed very frequently during video observation. Aggressive acts were mostly non-reciprocal, meaning that one animal was nipping or chasing the other, without any sign of defence by the chased animal. In the vast majority of observations, it was only one single animal that attacked all other animals in the tank. Reciprocal aggressive bouts between two fish were observed rarely compared to non-reciprocal aggressive acts. Those were very clearly distinguishable from non-reciprocal aggressive behaviour, as the fish circled each other, attacking each other frequently. The role of the attacker performing non-reciprocal aggressive acts was permanent, meaning that once an animal gained this position it stayed in this hierarchical superior position until the end of the trial. It also happened that the attacker got sick and died, in which case his position was taken by another animal of the group shortly before the attacker succumbed to disease. During the day, the

attacker was frequently observed hovering in the centre of the tank or changing location slowly and repetitively, often following a circular trajectory around the tank, as if patrolling. These fish are called dominant in the further course of this text. The fish that were not observed performing aggressive behaviour (usually all but the dominant animal) are designated as subordinate animals. During day-time the subordinate animals tended to aggregate at one location inside the tank. This could be for example near the surface or the bottom of the tank. In other cases the subordinate fish formed a tiny shoal in one corner of the tank. In AsT/T2 the subordinate fish always aggregated near the transparent glass pane, which divided two tanks. Fish dropping out of this tiny shoal were often immediately attacked by the dominant animal. This was most frequently observed in fish that were soon about to die, as they regularly exhibited disoriented swimming behaviour or the tendency to separate from the group. It could be observed that those dying fish were attacked most persistently by the dominant fish. Aggressive behaviour was almost exclusively observed during time of illumination (7:00 a.m. to 7:00 p.m.). During the night and during feeding only few acts of social aggression were observed.

3.2.8 Graphical analysis of spatial distribution

The graphical analysis of spatial distribution (see Fig.3.22 and Fig.3.23) overall confirmed the impression that arose during video-observation: during a 24 hours cycle the fish showed different patterns of spatial distribution. At time of illumination most movement was marginalised or concentrated at one single location of the tank close to the tank walls, either near the surface or near the tank bottom, while during the night, movement was distributed more evenly among the tank, although there were differences between the tanks regarding the degree of dispersion. This suggests that during the day, the fish seek proximity to each other and to the spatial borders of the tank (here called marginalisation), while during the night this tendency is observed to be less pronounced. Also, graphical analysis of spatial distribution affirmed the observation made by the author, that fish kept in opposite tanks separated by a transparent glass wall (AsT1/T2) acquired a mirror-inverted arrangement inside the tank, while fish in YrT1/T2 that have been equipped with an opaque separation did not.

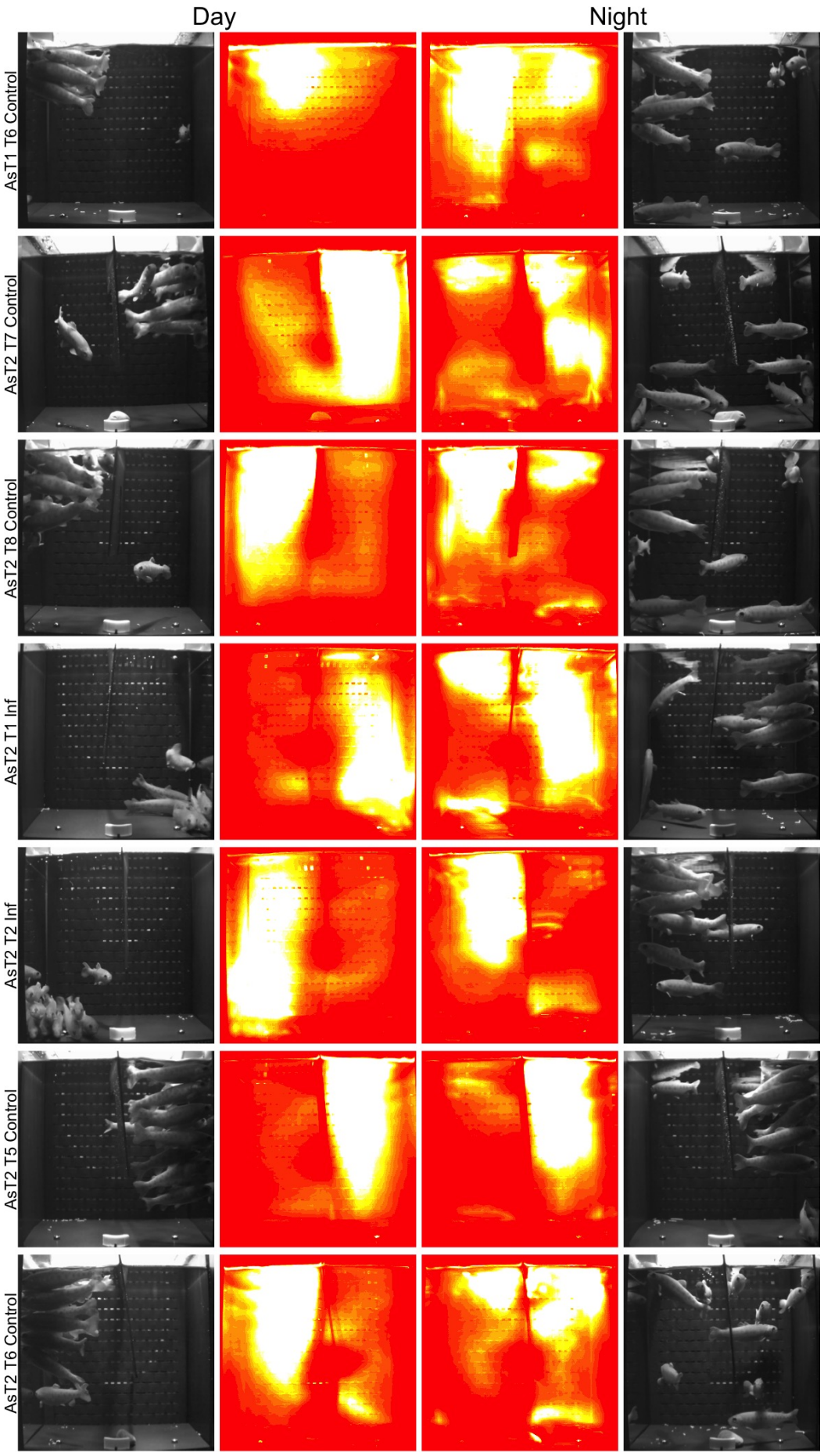

Figure 3.22: Graphical analysis ("Heatmaps") - AsT1/T2

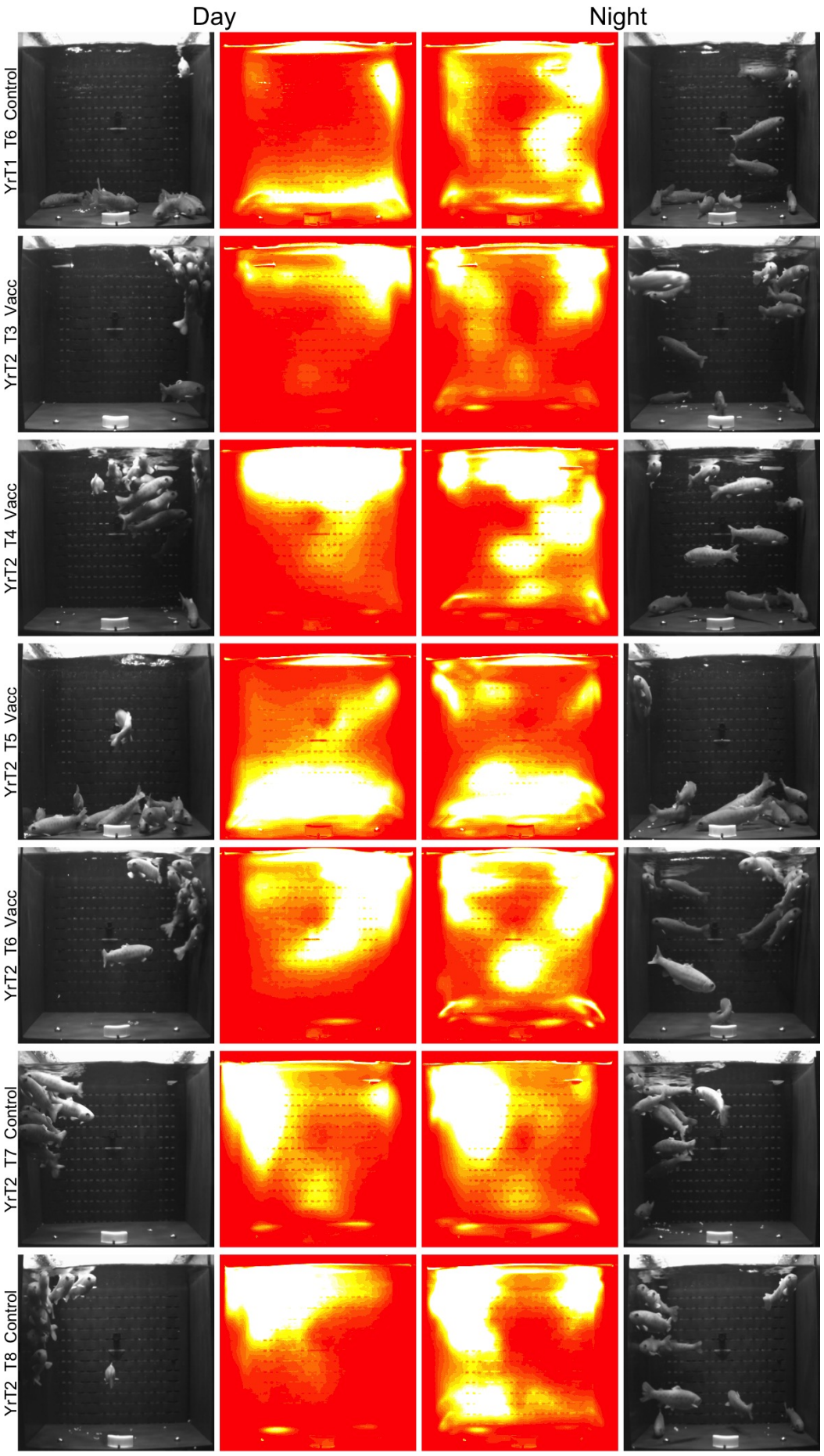

Figure 3.23: Graphical analysis ("Heatmaps") - YrT1/T2

Chapter 4

Discussion

The aim of this study was to identify visually perceptible indicators enabling early and reliable differentiation between survivors and nonsurvivors in two different infection trials in rainbow trout with help of video-observation.

Complete anorexia was thereby expected to be one of the most promising candidates. Anorexia is considered a typical sickness behaviour occurring in a broad variety of species and diseases. Loss of appetite, as a sequel to systemic bacterial, viral or protozoal infection is today thought to be an adaptive behavioural pattern, rather than the expression of a physiologically debilitated state [6, 23, 21, 5]. Alongside other behavioural patterns like lethargy and anhedonia, illness-induced anorexia is assumed to be the product of co-evolution between vertebrates and their pathogens, as it increased the probability of survival and reproductive success of the host (see [16, 6, 21, 5] for further reading). Research about the molecular mechanisms of sickness behaviour indicates that those behavioural patterns are mediated through a complex interplay of immune system, nervous system and endocrine system [6, 23, 21, 5]. This is thought to process internal and external information, in order to mount a behavioural response to infection that maximizes individual fitness[1] [5]. Being influenced by external stimuli, sickness-induced behaviours were found to be flexible to environmental conditions. Expression of sickness behaviour can be mitigated or enhanced by other factors than the degree of illness [6]. Aubert (1999) therefore describes sickness behaviour as reflection of a motivational reorganisation, while the motivational state still remains plastic to be influenced by stimuli that might be important for survival or reproduction. The universal occurrence of sickness behaviour in vertebrate animals is making its detection a widely used instrument in veterinary practice. Systematic assessment of partial or complete anorexia in big animal live-stocks has been proposed as a promising tool for early recognition of sick animals [44]. In many score sheets for assessment of health and well-being in laboratory animals, documentation of feeding behaviour is included (e.g. [27]).

Expression of sickness behaviour is thought to vary between species. In strongly territorial species, sickness behaviour is therefore assumed to be muted up to a certain extent, depending on internal and external conditions [5]. For a territorial species like rainbow trout, expression of sickness behaviour is likely to present a considerable cost. Its fitness highly depends on whether or not it succeeds to occupy suitable locations for effectively feeding on drift (i.e. macroinvertebrates that are carried with the water stream) while being hidden from potential predators. Field studies have shown that rainbow trout prefer locations that provide protection from above [30]. Partially muted sickness behaviour was thought to be advantageous for using anorexia as a death-predictor, because the author expected rainbow trout to show complete anorexia only in case of dramatic illness, eventually leading to death. Additionally, genetic selection for high growth-rates is thought to have increased the appetite of domesticated salmonid fish compared to their wild ancestors [14, 42]. Domestic rainbow trout were found to have higher blood plasma levels of Growth Hormone (GH) and Insulin-like-Growth-Factor (IGF-I) as well as expression of GH mRNA in the pituitary compared to wild rainbow trout [42]. Tymchuk et al. (2009) established the assumption that the high levels of IGF-I in domesticated fish might correlate with stronger

[1] Here the author refers to evolutionary fitness, not physical fitness.

appetite, which might be partly responsible for the higher growth-rates achieved by domesticated animals. Similar findings (elevated pituitary GH content and plasma growth hormone levels) were reported in domesticated Atlantic salmon [14]. This was rated as another factor rendering comlete anorexia a good predictor of severe illness in domestic rainbowtrout. Also, the binary quality (either a fish takes up food or it does not) of complete anorexia reduces observers scope of interpretation to a minimum, therefore it provides good observer reliability [19].

Complete anorexia presented one of the most sensitive and earliest PDP in this study. Once the nonsurvivors started to be anorectic, they stayed anorectic until death. Together with the relatively long forecast time, this provides a high probability of actually observing those fish, especially because the time of feeding can be determined by the observer. However, there were big differences in specificity between AsT1/T2 and YrT1/T2: while complete anorexia warranted reliable identification of nonsurvivors in AsT1/T2, the specificity was limited in YrT1/T2, due to the occurrence of complete anorexia also in survivors. Moreover, complete anorexia did not only occur in survivors, but as well in uninfected control animals and in vaccinated animals. Accounting the difference in specificity to the different pathogens is therefore considered unlikely. To the author, it seems more probable that this mismatch is caused by differences in feeding rate, in combination with a high degree of social aggression. Apart from illness-induced loss of appetite, social aggression might have presented a second cause for anorexia during this experiment.

The formation of a **dominance-subordination order** of despotic type (see behavioural observations described in Sec.3.2.7) could be detected during every trial, in every tank. The same phenomenon has been described frequently in studies that kept rainbow trout in small groups ($n \leq 25$) in relatively small aquaria (i.a. in [30, 46, 3, 25, 33, 38]). The tendency to form hierarchical social structures even makes the rainbow trout a suitable model species for examining the endocrine processes during social stress in fish (e.g. [45]). Under condition of spatial constriction and low absolute numbers of animals held in one tank, the effects of the social aggression were found to tremendously increase inter-individual variation. Two of the factors found to influence social aggression were: i) tank size: Newman (1956) reported higher number of aggressive acts (nibs per hour) in smaller tanks compared to bigger tanks; ii) number of animals per group: The lower the number of animals inside a tank (smallest group size being n=2), the stronger were deviations of physiological parameters in subordinate animals [33].

Morton (2001) emphasized how important it is to consider a species' biology, when pursuing refinement of animal experimentation. Rainbow trout are known to be territorial animals [30, 46, 22]. The species has been observed in the wild [30] as well as in captivity [46] to form "partial" territories: fish occupying a territory were observed defending it against smaller and equally sized fish, while being usually driven away by bigger animals. Newman (1956) reported from field observations in natural streams the existence of what he calls "rotating territories". He used this term to designate a highly dynamic situation, in which trout are circulating from territory to territory, chasing away smaller specimens, which themselves change location, either to occupy a temporary empty territory or to chase away another smaller animal from its territory. Overall, despite of forming shoals in cases of excitation in captivity, rainbow trout cannot be considered a social species in their natural environment. The high degree of social aggression observed during this and other studies is likely to be a consequence of the rainbow trout's natural behaviour of occupying temporary territories. But unlike under natural conditions, the subordinated animals cannot retreat under conditions of spatial confinement.

Being exposed to a high degree of **social aggression** has been proven to elicit intense physiological reactions in rainbow trout, especially in the subordinated animals. Pottinger and Pickering (1992) reported that subordinated animals showed chronically elevated cortisol levels, lower lymphocyte counts and higher mortality when compared to dominants, animals kept individually or animals held in high densities in spacious tanks. Other studies also demonstrated chronic elevation of blood cortisol levels in subordinate rainbow trout compared to single kept, or dominant animals [38, 45]. Subordinates were frequently found to experience weight loss [46, 33]. Social stress has been found to decrease feed intake in fish (see [8] for review). Within

the scope of behavioural observation studies, it was noted that severely dominated animals frequently displayed complete anorexia [30], showing no perceptible excitation when given food [46]. Although the exact mechanisms mediating anorexia as a response to social stress in fish are still to be elucidated, it is assumed that corticotropin-releasing-factor (CFR) is one substance mediating appetite-suppression in subordinate fish [8].

Therefore, the author thinks it is highly probable that the high level of social aggression observed during the experiment induced complete anorexia in fish that would have not shown anorexia under more species-appropriate conditions. A notable finding supporting this hypothesis is the **correlation between complete anorexia and fin or snout damage** in control and vaccinated animals of YrT1/T2. Severe abrasion of fins has been reported from other studies, in which fish experienced a high degree of social aggression and was observed to be caused by constant nipping of the dominant animal [46]. Constant nipping of fins by dominant animals (although not numerically assessed) was clearly visible during behavioural observation in AsT1 and YrT1/YrT2. In YrT1/T2 pathological examination revealed a high incidence of caudal fin damage. It was assumed that the degree of caudal-fin injury might reflect to a certain extent the amount of social stress experienced[2], as injuries are used as markers of social stress [9]. In AsT2 where tanks were equipped with PVC room divider to protect subordinates from the dominant, a big proportion of fish showed light to severe injuries of upper and lower jaw, while incidence of caudal fin damage was low. During behavioural observation it was noted that fish in AsT1/T2 had almost constant snout-to-glass contact (see also Fig.3.22, Chap.3), actively swimming against the glass. In the authors opinion, constant pressure on the relatively small area of skin and underlying tissue covering the apical tip of lower and upper jaw caused chronically reduced perfusion of the tissue, following necrosis and atrophy. The behaviour of swimming against the glass walls was not visible in dominant fish, except in moments when they seemed to attack fish on the other side of the glass wall, or in the attempt to catch food that was given in the neighbouring tank. In the authors opinion, the cranial lesions are therefore indirectly caused by social aggression and might be, too, correlated with the amount of social stress experienced by the subordinate fish.

But pronounced social aggression was observed during all trials, while increased proportion of anorectic survivors and control fish were observed primarely in YrT1/T2. The author assumes that the **high nutritional state** of fish in YrT1/T2 might have caused a higher incidence of complete anorexia. As displayed in Fig.2.2 fish assigned to YrT1/T2 received a higher amount of feed before and during the trial than fish assigned to AsT1/T2. This could have caused an effect on the manifestation of anorexia as there is scientific evidence that expression of sickness behaviour is in fact modulated by external and internal factors (see [23, 21, 5]. Leptin, a hormone produced by adipose tissue seems to have influence on the expression of anorexia and is assumed to influence sickness-induced anorexia [5] as well as stress-induced anorexia [8]. The author finds it likely that a high nutritional state of the fish in YrT1/T2 enhanced the manifestation of anorexia in infected animals, while anorexia in AsT1/T2 fish were muted until reaching a more severe degree of illness. Additionally, similar mechanisms might have been the cause of a higher incident of anorectic fish in the uninfected control group in YrT1/T2, as stress-induced anorexia and infection-induced anorexia might be mediated by similar endocrine (i.a. hypothalamic-pituitary-interrenal axis) and neurological pathways. Furthermore it was noted that in survivors, controls and vaccinated fish, the patterns of anorexia were strikingly different when compared to nonsurvivors. Animals often showed intermittent partial or complete anorexia, in contrast to long lasting complete anorexia demonstrated by nonsurvivors. While taking up food during the first feeding of the day, they showed low appetite or complete anorexia during the second feeding of the day. Because the interval between first and second feeding of the day was much shorter (7 hours) than between the second feeding and the first feeding of the following day (17 hours) it is assumed that increased appetite might have overruled social-stress-induced and/or illness-induced anorexia during the first feeding of the day. Additionally the wide absence of aggressive behaviour during the night

[2]Winberg & Lepage (1998) hypothesise that chronic stress is rather related to the threat imposed by the sheer presence of the dominant fish than by actual aggressive acts,as there is no positive correlation between plasma cortisol and number of attacks performed by dominant animals.

might have caused a reduction of social-stress-induced anorexia in the morning[3].

Because of the reasons discussed above, complete anorexia is still found to be one of the most promising death-predictors in infection trials with *A. salmonicida* and *Y. ruckeri*, despite the shortcomings in specificity in YrT1/T2. More research should be undertaken to examine the suitability of complete anorexia as a surrogate endpoint in infection studies with rainbow trout **under more species-appropriate conditions**. It is imaginable, that in single-housed trout, complete anorexia is much less likely to be expressed by survivors and control animals, reducing the false positive rate, because social stress is ruled out completely. This might enable reduction of animals in total, as inter-individual variation caused by social aggression would be decreased. Additionally, well-being of animals during experimental trial is thought to be improved tremendously, as lack of social interaction causes no activation of the hypothalamic-pituitary-interrenal axis (and therefore no stress) in this seemingly solitary species [45, 33, 38]. This might contradict the general chorus of keeping laboratory animals group housed [19]. In the author's opinion, there is enough scientific evidence, that *social housing* in rainbow trout in small groups in glass tanks constitutes rather a source of suffering than a measure of refinement. Whether social isolation elicits stress in an animal or not, is thought to be dependent on a species social organisation [9]. But as mentioned above, according to fields observations, most life stages of rainbow trout seem to avoid proximity to conspecifics. Although in the majority of performed infection studies, the number of animals kept in one tank might be greater ($\approx$ 30) than in this study, and the tanks might also be larger, it is still doubted by the author that social aggression is absent or can be considered negligible in terms of good scientific practice and animal-welfare. It is thought more likely that social aggression might not be recognised because of lack of awareness or because animals are mainly monitored during feeding or cleaning. In those situations the animals can be expected to display neither aggressive behaviour, nor the characteristic spatial distribution (marginalisation and/or aggregation of subordinates). Researchers conducting infection trials in small groups of rainbow trout should monitor their animals also outside the feeding times, as possible without being noticed by their animals, to check wether marginalisation and/or aggregation of subordinates is present while light coloured dominant fish patrol in the center of the tank.

The natural behaviour of these stream-dwelling animals, which spend big parts of the day holding position against the current and waiting for drift, could enable keeping them individually in relatively small, space-saving aquaria equipped with a customary aquarium-pump, without compromising their well-being. The pump could provide the positively rheotactic animals with a current, simulating more natural conditions and fulfilling their need for exercise under spatial confinement. Plasma cortisol of single-housed rainbow trout without a current should be compared to single-housed animals that have access to a current during acclimation, to examine a potential mitigating effect on stress levels. Ideally, the tank should be designed in a way that gives the animals the choice between a current-free area at the bottom of the tank and a medium strong current close to the surface, as this could facilitate identification of fish that show illness induced lethargy with settlement to the bottom. Fish could be kept on a low to medium feeding rate and fed several times per day to frequently assess presence or absence of complete anorexia.

Another major difficulty, when it comes to the identification of complete anorexia in rainbow trout, could also be solved by single housing: the visibility of single fish can be seriously limited by the high numbers of fish that are often kept together in one tank during infection studies [26]. Especially during feeding time, when the fish move inside the tank with high velocity, it is difficult for a human observer to identify lethargic or anorectic animals. In contrast to video-observation, it is not possible for an on-place-observer to rewind the tape, or set it on slow motion in order to recognise if a single fish is swimming restlessly without taking up food.

[3]Unfortunately individual weight and length were not measured in AsT1/T2 at the time of bacterial challenge, otherwise it would have been possible to compare body condition factor [Length(cm)/Weight(g)] between the groups. Fish from AsT2 had the highest median weight per tank (see Fig2.1 in Chap.3), however control animals of AsT2 had also the highest median body-length after end of experiment (AsT2=12.4 cm, YrT1=10.1 cm, YrT2=11.4 cm). Between YrT1 and YrT2, fish from YrT2 had the higher median body condition factor at bacterial challenge (YrT1=1.4, YrT2=1.62).

One morphological change that could be observed during the trials was a **change in body shape** in fish that have received an intra-peritoneal injection of *A. salmonicida*. The appearance of the S-shaped abdominal curvature (associated with kyphosis) in nonsurvivors can be explained by the presence of **peritonitis**. Pathological findings in those fish strongly indicate the presence of acute to subacute peritonitis. The rough parietal peritoneum is likely so be caused by fibrin deposits, which formed trough an inflammatory response in the peritoneal cavity. Extensive red colouration of the parietal peritoneum might be a consequence of vasodilatation and haemorrhage as part of an inflammatory response. Intraabdominal fluid (i.e. ascites) presents most likely inflammatory exsudate. Accumulation of inflammatory exsudate in the peritoneal cavity presents a common clinical finding in animals with peritonitis, leading to differing degrees of abdominal distension. A high proportion of fish in AsT2 had perforated body cavities, with abdominal walls being thinned out to such an extent, that they ruptured easily during handling of the dead animal. This is not thought to be caused by post-mortem autolytic processes, as fish being removed only short after dying ($1/2$-3 hours) also demonstrated those findings (water temperature $\approx 14°$C). Perforated abdominal walls were also apparent, but unfortunately not numerically assessed in AsT1, as no pathological examination was performed. Intact but focally reddened abdominal skin was found to be localised over areas of internally destructed abdominal wall. This creates the impression that progressive destruction of all layers of the abdominal wall was proceeding from inside the body cavity to outside. *Aeromonas salmonicida* has been found to have strong collagenolytic properties [13], explaining the extensive lesions of all anatomical layers of the abdominal wall. In our trial this even resulted in complete loss of integrity and opening of the body cavity in some of the animals. The localised bulging[4] of the abdominal wall seen in AsT1/T2 might also be caused by nearly perforated abdominal wall, giving away to increased intraabdominal pressure by fluid accumulation and increased tension of abdominal muscles.

A typical finding observed in a wide range of animals with peritonitis (humans included) is a **reflective tension of the abdominal muscles** [20, 7, 36]. In terrestrial mammals, this can be assumed to be the consequence of inflammation of the parietal peritoneum as chemical, mechanical or temperature stimuli elicit strong pain reactions, in contrast to stimulations of the visceral peritoneum [31]. The parietal peritoneum of the fish of AsT2 showed pathological alterations indicative for severe inflammation. In the authors opinion, the reflective tension of the abdominal muscles, together with varying degree of fluid accumulation inside the peritoneal cavity, is likely to have caused the S-shaped abdominal curvature observed. This is further supported by the finding that also the dorsal shape of clinically sick nonsurvivors was found to be slightly more convex compared to surviving animals. The expression of kyphosis could be explained by the increased tension of the abdominal muscles, which cause the vertebral column of the fish to bend dorsally. This clinical sign, which is often called “arched back”, is a frequently observed symptom in tetrapod terrestrial animals that suffer from peritonitis.

Pottinger & Pickering (1992) mention that in pairwise kept trout, the subordinate fish often demonstrated an “unnaturally bent body posture” while remaining at the tank bottom. This behaviour was also observed by Abbott et al. (1985) and called “hunching”. It could also be observed during this experiment, but it is not considered to be identical with the kyphosis shown by the animals with peritonitis. Hunching could only be observed in animals sitting at the bottom of the tank, while kyphosis was also demonstrated while swimming. Kyphosis was also not connected to an immediate threat by a dominant fish, as it was also observed during night time, when the dominant animals showed little to no aggressive behaviour. It is suspected by the author that hunching might present a “defensive posture that is seen primarily in inescapable situations” [9] and should not be confused with kyphosis.

An objective confirmation about the subjectively perceived change in morphology was brought by graphical analysis of abdominal and dorsal curvatures. It proved that, a few hours before death, nonsurvivors demonstrated indeed a more S-shaped abdominal curvature compared to the surviving animals. It has to be emphasised, that the change in the abdominal shape was not solely found to consist of an abdominal distension, although pronounced abdominal distension

[4] Photo B.9 given in Appx.B

could be observed. However, a simple increase in abdominal circumference, while remaining an U-shape could be seen in surviving animals. This changes in abdominal curvature in the survivors might be caused by the higher amount of food that is consumed by the surviving fish, as in this study the nonsurvivors were found to cease feeding, leaving their portion of food to the surviving animals. The well filled intestinal tract of the latter is assumed to have lead to the observed U-shaped abdominal distension. The occurrence of abdominal distension on both survivors and nonsurvivors could present a problem regarding the practical applicability of this PDP as a surrogate endpoint. Correct identification of each shape might need considerable training, observation time and good observational skills. To further explore applicability, it would be necessary to determine intra-/inter-observer reliability, before and after training.

What might render evaluation of abdominal and dorsal curvature less suitable as a surrogate endpoint is that it was frequently not assessable (50Q(NaObs%)=62.5%). Deciding between presence and absence of this PDP required clear visibility of the respective fish, lateral display and a central position in front of the camera to avoid perspective distortion. This was often not the case during observation. Additionally, abdominal S-shape was felt to develop gradually and the degree of expression was felt to vary strongly between individual fish, with the tendency of fast dying fish to develop this PDP stronger and more reliably. In fish showing low to intermediate degree of an S-shaped abdominal curvature it was often difficult to decide between presence or absence of a tucked-up abdomen. This is also reflected by the comparably low intra-observer reliability, which was the lowest of all tested PDPs. However, this could be also due to the special experimental conditions. As the light source was installed *above* the tank, the fish's bellies were frequently cast in shadow, which was why it was often impossible to differentiate a fish's abdominal silhouette from the dark background. It might be possible that direct observation would have resulted in better visibility of this trait. Regarding median forecast time, a tucked-up abdomen presents a favourable candidate for a surrogate endpoint, as it enables comparably early identification.

Deviation from normal locomotion was observed in AsT1/T2 as well as in YrT1/T2 and designated as "stiffened locomotion", although there is some doubt on the side of the observer, if the observations in AsT1/T2 and YrT1/T2 were in fact identical or just very similar. Indeed a fish's movement pattern, which is in principle a sequence of morphological changes, represents a very difficult issue to be visually analysed by a human observer, due to its dynamic and complexity [5]. Of course the difficulty decreases with increasing degree of deviation from perceived normality. The presence of a current in the tanks of YrT1/T2 in contrast to AsT1/T2 might render it actually illegitimate to regard the stiffened locomotion in AsT1/T2 and YrT1/T2 as identical phenomenon, as absence or presence of a current can be expected to have crucial influence on a movement pattern. Instead they should be regarded as two similar locomotive patterns, which were perceived to deviate from normal locomotive pattern observed in each tank.

In AsT1/T2 stiffened locomotion presented a more suitable PDP than in YrT1/T2. The abnormal movement pattern among this group is thought to be linked to peritonitis, with the accompanying clinical signs already discussed above (S-shaped abdomen, kyphosis). The reflective tension of abdominal muscles might have interfered with normal latero-lateral, undulating movement of the cranio-caudal body axis, or rather, the reflective tension of muscles might be triggered to avoid movement at all. Human patients with peritonitis are described to avoid any kind of movement [36] presumably to prevent pain elicited by mechanical stimulation of the inflamed parietal peritoneum. An additional or alternative explanation might be the presence of meningitis in the animals infected by *A. salmonicida*. Histopathological examination in rainbow trout injected with extracellular products of *A. salmonicida* found oedema, haemorrhage and infiltration by lymphocytes into the meninges in the area of the optic lobes [13]. Altered movement patterns could therefore also, or additionally, be caused by neurological dysfunction. This hypothesis is supported by the finding that in 77.5% of all observations (in AsT1/T2), in which collision with tank walls (VPT = Col, see Tab.3.2) was observed, stiffened locomotion

[5] It is known from veterinary practice that it takes immense experience and expertise to correctly diagnose lameness in horses or dogs, a lameness constituting a deviation from normal movement pattern.

was apparent at the same time. A possible explanation for collision with the tank walls might be neurological impairment in form of blindness, caused by meningitis.

Settling motionless on the bottom of the tank was observed in the majority of nonsurvivors in AsT1/T2 where it was chronologically closely linked to lateral and dorsal recumbency. Overall its suitability as death predictor does not seem convincing, especially not in YrT1/T2. One can raise again the question, whether the prevailing conditions of this experiment were prohibiting the expression of this PDP. Rainbow trout where shown to demonstrate reduction of swimming activity as a consequence of nociception [39], which might have been caused by peritonitis in AsT1/T2 fish. Also, reduced physical activity is one of the cardinal sickness behaviours [16, 6, 21, 5]. Therefore settling motionless to the bottom of the tank seemed to be a promising indicator of disease in those infection studies. Two factors might have interfered with expression of this behaviour: i) social aggression in all trials; it was observed several times, that dying fish that separated from the other subordinate fish were vigorously attacked by the dominant animal. It is possible that nonsurvivors would have settled more frequently and earlier before death to the bottom of the tank, if they were not constantly attacked by the dominant animals. And on the other hand, graphical analysis of the spatial distribution showed cases, where all the subordinated fish where permanently localised at the bottom of the tank. As this is assumed to be a consequence of social aggression, social aggression might be indirectly held responsible for diminishing sensitivity and specificity of this PDP; ii) current in YrT1/T2; its possible, as current-free area at the bottom of the tank was quite limited in YrT1/T2 that fish were not able to settle down on the tank bottom as in AsT1/T2. Even if the current was weak, it still necessitated swimming activity to keep a stationary position. So despite the results of this study, which indicate that settling motionless to the bottom of the tank is only a mediocre to unsatisfying death predictor, it should not be discarded, but tested again under different conditions.

Dorsal rebumbency, lateral recumbency, severe perpendicular instability, light perpendicular instability and passive floating with the current are traits that correspond most to the general idea of a so called "moribund fish". The term *moribund* is regarded as problematic by the author, due to its inaccuracy when it comes to practical application [41, 15] and is therefore avoided in the further course of this text. However, aforementioned PDPs are chronologically closely linked to death and fit the description of the moribund condition by Toth (2000) of a "severely debilitated state that precedes imminent death". Those PDPs predict death in a highly precise fashion, as they showed high sensitivity, specificity and a low variation in forecast time. Nonetheless, their application as surrogate endpoint cannot be considered efficient, out of two reasons: i) short forecast time; in the vast majority of fish observed, forecast times ranged only couple of hours, meaning that the time an animal spends inside the experiment, which is possibly connected to suffering, would not be effectively reduced. ii) short duration; even though those PDPs were present consistently, from their onset to death, the duration can be considered much too short to be effectively detected by routine daily observation inside a laboratory. In order to do so, the animals would have to be monitored at least every hour.

In contrast to other PDPs examined in this study, dorsal rebumbency, lateral recumbency, severe and light perpendicular instability and passive floating with the current are not thought to be strongly influenced by the interior design of the tank or other environmental conditions of this study. Although, it might be possible that the current inside the tanks of YrT1/T2 might have caused the fish to show those traits a little later in time than the animals of AsT1/T2, because the current provided them with a stimulation to swim. But still, the external validity for those PDP is estimated to be quite high. They are considered to express the inability to show normal movement or behaviour, which is caused by debilitation of the physiological state of those animals, presumably by multiorgan failure and therefore (unlike sickness behaviour) not thought to be strongly influenced by environmental factors.

Overall darkening of skin color is an unspecific clinical sign, which is described commonly in many different diseases in fish [32]. Interestingly darkening is also observed in subordinate salmonid fish, which might be connected to increased expression of pro-opiomelanocortin (POMC) in the pituitary of subordinated rainbow trout [45], while dominant fish tend to be light

in colour [30, 46]. Although it was thought that differences in brightness would not be observable under the present technical conditions, it was found to be regularly detectable by the author and therefore cannot be ruled out as a suitable PDP, especially in the absence of social interaction.

Another of the clinical sings that were observed, but not systematically assessed was an **increase in frequency of opercular movement**. This could be observed especially during the night-time, when overall activity of fish was lower and opercular movement could be compared among the animals in the tank. Two common aetiologies thought to increase opercular movement in rainbow trout are oxygen deficiency (environmental or caused internally e.g. by anaemia) and pain [39]. In the fish infected with *Y. ruckeri*, increased ventilation rate can be most plausibly explained by the presence of extreme anaemia. Pathological findings in those fish were typical for a haemorrhagic septicaemia caused by systemic infection with *Y. ruckeri*. As *Y. ruckeri* is known to cause necrosis in haematopoetic organs in fish [32], reddened swimbladder, pink coloured perivisceral fat as well as reddened parietal peritoneum in proximity to the spleen might be caused by haemolytic imbibitions from kidney and spleen. Reddened skin seen on the head and in the oral cavity indicate typical haemorrhages that are frequently reported in *Y. ruckeri* outbreaks [32]. Extremely pale gill and liver colouration strongly suggest that fish suffered from massive anaemia. Flared opercula and opened mouth in 5 of 14 dead fish are considered a diagnostic sign, indicating that fish suffered from oxygen deficiency prior to death [32], which can be explained by impaired oxygen-transport accompanying the massive anaemia. In consequence, internal oxygen-deficiency caused by anaemia is considered the cause for the increased ventilation rate observed in nonsurvivors.

In the fish infected with *A. salmonicida* pronounced clinical signs of anaemia in form of pale gills or internal organs could generally not be observed by the bare eye, although it has been shown that extracellular products of *A. salmonicida* posses haemolytic activity. It has to be noted, that a reduction in blood erythrocytes or haemoglobin is detectable by visually evaluating the colouration of mucosal skin only after dropping beneath a certain level. That the higher respiratory rate is caused by internal oxygen deficiency can therefore not be excluded, as no determination of haematocrit was performed. However, a mild anaemia might have not caused symptoms of oxygen-deficiency. Another possible reason for the increased ventilation rate in this disease model might be stimulation of nociceptors located in the parietal peritoneum by the massive peritonitis observed in these fish. Peritonitis is known to be an extremely painful condition in humans. Significant increase in respiration rate could be observed in rainbow trout receiving injections of acetic acid and is thought to be a reaction to nociception or pain [39]. Increased ventilation rate, although not numerically accessed in this study might be a valuable PDP in those two disease models. Of course only under conditions of sufficient water oxygenation and good visibility of individual fish, which might be difficult in case of high numbers of animals within one tank or enhanced activity due to excitement, because this might increase ventilation rate also in healthy fish as well as reduce visibility of individual animals.

Changes in ventilation rate may even provide opportunity for using an automated technique used for continuous effluent acute toxicity surveillance [37]. The technique resembles electromyography (EMG), a medical technology for measuring electric muscle activity. In this case too, single-housing would be of advantage. Eventually usage of this technique would necessitate a pump that is attached outside the aquaria to not disturb the electrical signals emitted by the fish's muscular activity. Provision of an unidirectional water current would warrant a constant alignment of fish in one direction and might facilitate precision of this method.

Nonsurvivors in all four trials were occasionally observed to show **emesis** (vomiting). In YrT1, emesis was even observed in surviving animals. Although this might be an interesting discovery, short duration and rare occurrence of this clinical sign renders it largely unusable as a surrogate endpoint.

In the following, some comments are added about different factors that might have had undesirable effects in this study. One of them was the **different origin** of the rainbow trout used in those trials. The informative value of this study might have been improved by distributing the fish of both origins equally among the four trials, so that all trials contained fish of both

origins. However this would have meant an even higher variation in body size inside groups (i.e. increasing intra-group variability of body size). On the other hand using fish of one and the same batch for all four experiments would have led to increasing body sizes from trial to trial (i.e. increasing inter-group variability of body size). It seemed impossible at that time to predict the consequences either solution would have had in terms of social interactions between the trout. There was also no consent, whether there could be differences in susceptibility between different strains of trout. It has to be mentioned that rainbow trout do not reproduce continuously over the year, so obtaining animals of the same age and size at different points in time can constitute a difficulty.

Further, fish used for YrT1/T2 were submitted to antibiotic treatment before they were infected with *Y. ruckeri*. It could be argued, that this might have influenced the course of disease and respectively clinical signs shown by the animal. But considering the 1046 (YrT1) or 1256 (YrT2) day degrees between end of treatment and beginning of experiment, it can be assumed that this treatment didn't affect the course of infection, as withdrawal period for food-fish after treatment with a similar product (Aquaflor ®), containing Florfenicol as active ingredient, is indicated as 135 day degrees.

Because the provisional internal design of the tanks made cleaning procedures, especially removal of biofilm infeasible, fish in this experiment experienced **no acclimation period** inside experimental tanks. When considering the high intensity of agonistic behaviour and the sometimes considerable injury observed in the animals, it can be questioned weather more time under experimental conditions would have been justifiable in terms of animal-welfare. Still, the lack of acclimation has to be noted, as it can be considered as a source of confounding.

One type of observation that has been largely missing in this study, is **provoked behaviour**. Cameras were mounted inside black plastic boxes in front of the aquaria to avoid reflections. Thus it was impossible for the fish to see persons standing or moving in front of the aquaria, although it might have been possible that the fish were able to perceive vibrations by footsteps. Under usual circumstances rainbow trout can visually precept the presence of persons, provided that animals are being kept in glass aquaria. They show behavioural changes in reaction to disturbance e.g. in form of flight, formation of swarms or they swim restless at the water surface, presumably in expectancy of food. Because of the technical setup, alterations in the reaction to observer presence could be judged only to a small extend, which was only a few seconds before feeding, as the fish reacted to the shadow cast by the author's hand while opening the aquarium lid. This might have eliminated one source of PDPs, as abnormal reaction to observer presence or handling is regarded as valuable signal in terms of well-being [27]. On the other hand, video-recording might have facilitated detection of behaviour that would have been possibly muted in the presence of an observer, a phenomenon that is been tried to be avoided by automated measurements [44].

Conclusions and perspectives

Video-based observation can be considered a valuable tool for assessing surrogate endpoints in fish infection experiments. It enables retrospective and repeated observation and therefore higher utilization of a single trial than direct observation. Thanks to the availability of modern surveillance technique, high quality videos can be produced by non-professionals. Planning and installation are yet connected to some effort that might render video-based observation unsuitable for many institutions conducting infection experiments with fish. However, availability of a video-recording-system might strongly facilitate systematic observation and consequent identification of surrogate endpoints, as it gives the observer a high degree of flexibility and comfort.

It is assumed that clinical signs comprising a change in morphology (e.g. S-shaped abdominal curvature), or clinical signs that indicate a highly debilitated state (e.g. lateral or dorsal recumbency, perpendicular instability) are likely to be expressed similarly under differing husbandry conditions and can therefore be considered to be of high external validity. But potentially more efficient death predictors, like complete anorexia are likely to have been severely affected by the high degree of social aggression that was evident throughout the trials. More research should

be conducted to evaluate those promising PDPs under experimental conditions controlling for social aggression. Indeed, variability in behavioural patterns shown by survivors and expression of clinical signs in nonsurvivors can both be expected to be much smaller in single-kept rainbow trout. This would enable earlier distinction between surviving and non-surviving fish. Therefore, application of surrogate endpoints is suspected to be much more reliable and efficient under social isolation. It is also assumed to increase overall welfare by eliminating social stress experienced by the subordinate rainbow trout.

However, as long as numbers of fish remain high in efficacy tests and husbandry conditions are not precisely defined, investment in this direction of research might be questioned. The author thinks that successful application of surrogate endpoints in those experiments is only possible as part of an overall strategy targeting reduction of inter-individual variation by optimized husbandry conditions and aiming at limiting animal numbers to a statistical sound minimum.

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C. Keeling

Appendix A

Supplementary Tables

Figure A.1: Overview - number of surviving, nonsurviving and positiv re-isolated fish

AsT1					**AsT3**						**YrT1**						**YrT3**					
Tank ID	K	Group	d	s	Tank ID	K	Group	d	s	rpos	Tank ID	K	Group	d	s	rpos	Tank ID	K	Group	d	s	rpos
T1	K15	Inf	4	6	T1	K5	Inf	4	6	1	T1	K13	Inf	0	10	0	T1	K6	Inf	8	2	0
T2	K13	Inf	6	4	T2	K6	Inf	1	9	0	T2	K14	Inf	8	2	0	T2	K13	Inf	6	4	2
T3	K8	Inf	7	3	T3	K15	Inf	3	7	0	T3	K16	Inf	4	6	1	T3	K14	Vacc	1[1]	9	0
T4	K7	Inf	5	5	T4	K16	Inf	2	8	0	T4	K7	Inf	5	5	0	T4	K15	Vacc	0	10	0
T5	K14	Inf	9	1	T5	K7	Contr	0	10	0	T5	K8	Inf	4	6	2	T5	K16	Vacc	0	10	0
T6	K16	Contr	10	0	T6	K8	Contr	0	10	0	T6	K15	Contr	1[3]	9	0	T6	K5	Vacc	1 [2]	9	0
					T7	K13	Contr	0	10	0							T7	K7	Contr	1[2]	9	0
					T8	K14	Contr	0	10	0							T8	K8	Contr	0	10	0

[1] Euthanasia because of complete abrasion of caudal fin.
[2]Suspected swim bladder stress syndrome.
[3] Death during narcosis.

Tank ID	Experiment-specific Tank ID.
K	Facility specific Tank ID.
Inf	Infected group.
Contr	Uninfected control group.
Vacc	Infected vaccinated group.
d	Animals which died or were euthanized during the trial.
s	Animals which survived during the trial
rpos.	Animals in which A.salmonicida or Y.ruckeri could be reisolated from the head kidney.

Appendix B

Photos and Illustrations

Figure B.1: Technical set-up - External view : AUER®boxes

Figure B.2: Technical set-up - External view : ABUS®infrared spotlights.

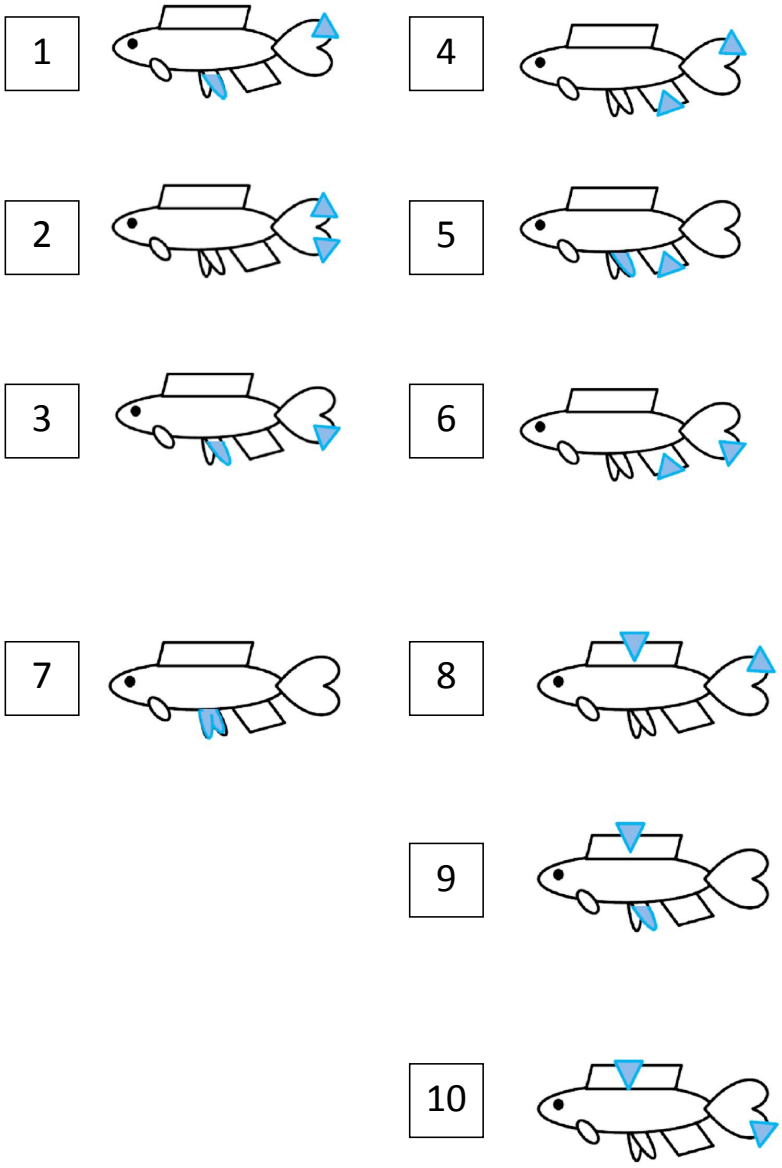

Figure B.3: Fin-clipping scheme utilized for tagging individual fish.

Figure B.4: Gross pathology - AsT2; nonsurvivor, which died after injection of *A. salmonicida* into the peritoneal cavity, shows highly reddened parietal ans visceral peritoneum and rounded spleen.

Figure B.5: Gross pathology - YrT1/T2 ; Upper photo shows fish with extremely pale gills. Lower photo shows the extremely pare liver, water filled stomach and the reddened swim bladder that were frequently seen in the fish succumbing to *Y. ruckeri* infection

Figure B.6: Gross pathology - AsT2; fish euthanised after end of infection trial. Severe cranial lesions (Sn=3) assumed to be caused by pressure atrophy.

Figure B.7: Gross pathology - YrT2; caudal fin damage degree 3 (FD=3) in fish euthanised after end of infection trial.

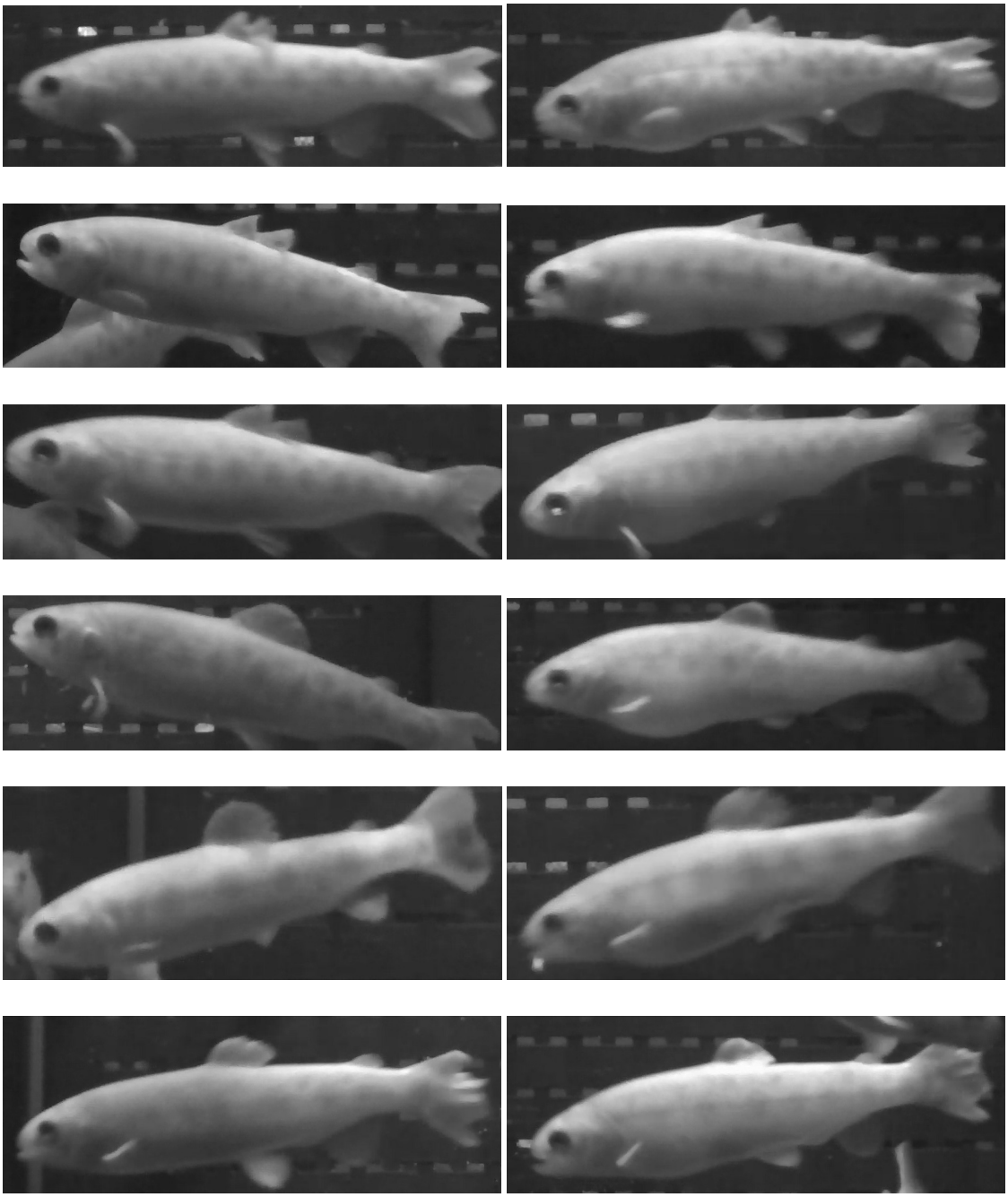

Figure B.8: TAbd - AsT1/T2; Snapshots showing the same fish directly after bacterial challenge (left row) and few hours before death (right row).

Figure B.9: Fish, which had received intraabdominal injection of *A. salmonicida* demonstrating localized buldging of abdominal wall.